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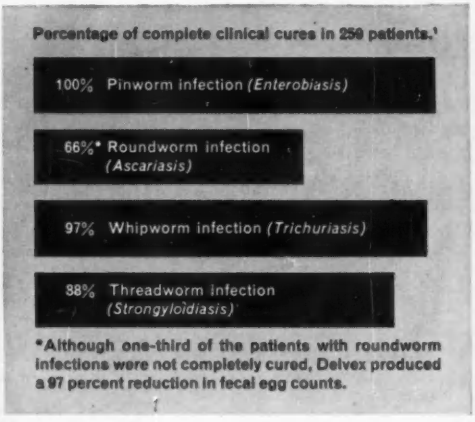
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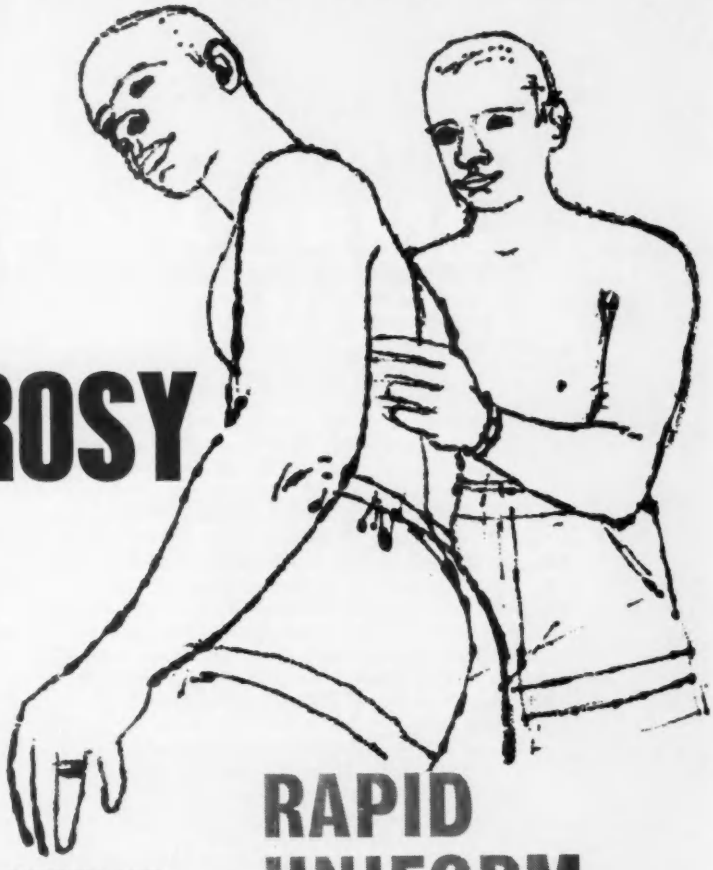

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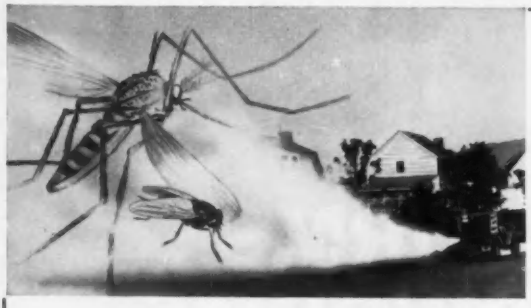

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CHLOROQUINE TREATMENT FOR MALARIA IN SEMI-IMMUNE PATIENTS

D. F. CLYDE

Malaria Service, Ministry of Health, Tanganyika

In Central Africa, Stephens and Christophers[1] concluded that malaria parasites could be driven rapidly and completely from the peripheral circulation of indigenous people by using as little as 10 grains (670 mg) of quinine. But the reluctance of physicians to administer what appeared to be marginally effective doses of quinine in so serious a disease as subtertian malaria, together with the fear of blackwater fever, has tended to carry over to the newer synthetic compounds. This applies both to the treatment and the suppression of malaria among semi-immune people.

When chloroquine is used, the single-dose dispensary treatment of semi-immune patients is widely favored, and for suppression a weekly or fortnightly regimen. The dosages in terms of base recommended by Covell *et al.*[2] are, for treatment of children 0 to 1 year, 37 to 75 mg; 1 to 3 years, 225 mg; 3 to 6 years, 300 mg; 6 to 12 years, 450 mg; and for adults, 600 mg. For suppression, the respective dosages are 37 mg, 75 mg, 100 mg, 150 to 225 mg, and 300 mg.

The investigations recorded in this paper show, however, that these doses are unnecessarily high for semi-immune patients in East Africa.

METHODS

The chloroquine trials described here were carried out in holoendemic malarious districts of Tanganyika, principally in the vicinity of Muheza. Semi-immune Africans reared in these districts, the endemicity of which has been fully described by Davidson and Draper,[3] were treated directly by the writer. All four species of parasite are present, with *Plasmodium falciparum* greatly predominating.

For the clinical trials, semi-immune patients complaining of fever, and found to have signs consistent with a diagnosis of malaria, were treated with chloroquine phosphate. Small children received the drug in a granular preparation, each granule containing 1 mg base chloroquine coated in hard sugar. The diagnosis of malaria was based on the presence of signs, together with a parasite count raised above the level expected of asymptomatic carriers in the area of holoendemicity. In addition, a refinement was necessary in the microscopic diagnosis of malaria: only cases having young filamentous rings, or schizonts, and not those with pigmented half-grown trophozoites, were accepted as being clinical malaria. Because of the high degree of transmission in the district, the many previous attacks of malaria suffered by the inhabitants, and the concurrent parasitemia and splenomegaly of many people not apparently ill, some of the clinical signs usually regarded as essential to the diagnosis of malaria were inconstant or unreliable.

For the suppressive trials, semi-immune carriers of malaria were treated with sufficient chloroquine to clear parasitemia and were then given varying doses in order to determine the smallest quantity required to keep the blood clear. In the districts concerned, it has been calculated that transmission is at the rate of one infective bite each night, and the inhabitants rarely use methods of personal protection against anophelines.

RESULTS

The results are recorded in three sections, as follows:

1. The smallest dose of chloroquine successful in clearing asexual parasites of all species from the blood of semi-immune patients suffering from clinical malaria. While the dose given was always effective among the patients treated in this investigation, in a larger series a failure might have been encountered. The dose is therefore described as the therapeutic ED_{99+}.

2. Clinical aspects of the therapeutic ED_{99+} compared with greater dosages, with reference to (a) toxic side effects; (b) rapidity of remission of fever, symptoms, and parasitemia; and (c) radical cure of subtertian malaria.

3. The smallest dose effective in keeping the blood free of parasites of all species, once they have been cleared. This may be called the suppressive ED_{99+}.

TABLE 1

Single dose chloroquine treatment of semi-immunes suffering from malaria, showing patients cleared of asexual parasites within 7 days, patients vomiting within 3 hours of treatment, and clearance time in hours

	Single doses in mg base chloroquine					
	9	19	37	75	120	37–300 scale
Age: 0–1 year						
No. treated	3	12	8			8
No. cleared	1	12	8			8
No. vomiting	0	0	0			0
Clearance time		41	44			44
Age: 1–2 years						
No. treated		10	19	11		11
No. cleared		5	19	11		11
No. vomiting		0	0	0		0
Clearance time			36	39		39
Age: 3–5 years						
No. treated		2	7	15		10
No. cleared		0	5	15		10
No. vomiting		0	0	0		1
Clearance time				41		37
Age: 6–10 years						
No. treated		11	21	32	18	12
No. cleared		4	18	31	18	11
No. vomiting				0	0	3
Clearance time				32	38	35
Age: 11–15 years						
No. treated			12	16	15	8
No. cleared			9	14	15	8
No. vomiting				0	0	1
Clearance time					34	37
Adults						
No. treated			9	14		9
No. cleared			7	14		8
No. vomiting			1	0		2
Clearance time				33		34

1. *The Therapeutic ED_{99+}*

It was found in earlier trials that single doses of 75 mg chloroquine base were almost always sufficient to clear asexual parasitemia among semi-immune children.[4] For the purpose of the present investigation, semi-immune patients with confirmed malaria attacks have been treated with the following single doses of chloroquine phosphate (recorded as base), and their blood examined for parasites 7 days later: 9 mg, 19 mg, 37 mg, 75 mg, and 120 mg. In addition, a contrasting series has been included using the larger dosage scale recommended by Covell *et al.*[3] and described in the introduction to this paper.

The results of the single dose treatments are recorded in Table 1. It will be noted that the therapeutic ED_{99+} was 19 mg for infants less than 1 year old, 37 mg for children 1 to 2 years old, 75 mg for those 3 to 5 years old, and 120 mg thereafter.

2. *Clinical Aspects of the Therapeutic ED_{99+}*

Toxic side effects. A quantity of chloroquine that would not produce adverse symptoms in a well-nourished non-immune patient being treated in bed might not be so innocuous to a native who has walked some distance to the dispensary, taken the medicine, and walked home where he may, or may not, be able to rest. Many of the patients among whom this work was carried out were infected with hookworm, roundworm, vesical bilharziasis, and bancroftian filariasis; some were tubercular, and many markedly anemic.

Assessment of minor degrees of drug toxicity is difficult in the presence of a disease with symptoms as varied as those of malaria, particularly among patients usually victims of one or more of the conditions noted above. For example, complaints of giddiness and collapse following treatment, often on the road home from the dispensary, were found upon careful inquiry not to exceed complaints of giddiness and collapse on the road towards the dispensary. The only objective indication of lack of tolerance to chloroquine, on which reliance could be placed, was vomiting occurring within 3 hours after drug ingestion. In Table 1 is shown the incidence of vomiting following treatment among patients not so afflicted prior to treatment. It is apparent that the number is low. Treatments were generally given during the morning, when most Africans have as yet eaten little.

The results indicate that the greater the dosage, the more likely is vomiting of the drug.

Rapidity of removal of fever, symptoms, and parasitemia of all species. An important factor in the choice of an antimalarial drug is the speed

with which it rids the patient of fever and attendant symptoms, and of parasitemia. In this investigation, temperature readings taken every 8 or 12 hours revealed that fever attributable only to malaria, and not to an underlying pulmonary or other condition, was relieved in most cases within 24 hours by any dosage capable of clearing parasitemia. The few exceptions persisting up to 36 hours were equally divided between patients on the large dosage scale and those on therapeutic ED_{99+}. Symptomatic improvement could not be assessed accurately in view of the diversity of complaints, some undoubtedly not related to malaria. In general, relief of symptoms appeared to lag some 12 hours behind relief of fever, irrespective of the size of the chloroquine dosage employed.

The speed of clearance of asexual parasitemia was determined more accurately, blood slides being taken at 8- or 12-hour intervals until two consecutive slides were negative. The results are shown in Table 1 ("Clearance time"), and it is apparent that there was little difference in the times, 32 to 44 hours, whatever the dosage used, the age of the patient, or the species of parasite.

Radical cure of subtertian malaria. In the case of subtertian malaria, a dosage of chloroquine sufficient to obtain radical cure is desirable. It is of course difficult in endemic malarious areas to decide when radical cure has been achieved in a semi-immune patient, for upon discharge he returns to his home and is at once exposed to fresh infection indistinguishable in the blood from recrudescence. In the present trial, a radical cure of subtertian malaria was considered to have been obtained if the blood of the patient remained free of trophozoites for a period of 3 months, and was determined thus: a small group of semi-immune school children 9 to 15 years old, native to a district of holoendemicity and infected there during the vacation, attended school as boarders in a mountainous malaria-free district. Those with subtertian malaria on their return to school received the single dose treatment recorded in Table 2 and were then observed for the remainder of the term, that is, for 3 months.

Provided it is accepted that a period of 3 months free from recrudescence of *P. falciparum* represents radical cure, it appears in Table 2 that the large dosage scale of Covell *et al.*[2] and the therapeutic ED_{99+} are equally effective.

TABLE 2

Radical cure of subtertian malaria in semi-immune children 9 to 15 years old

	Single doses in mg base chloroquine	
	120	37–300 scale
No. treated.....	7	5
No. cleared.....	7	5
No. remaining clear for 3 months.....	7	5

TABLE 3

Suppression of malaria among semi-immune people

Age in years	No. treated	Percentage of people protected by the following doses in mg base chloroquine			
		9	19	37	75
0–1	20	33	100	100	
1–2	*40		42	100	100
3–5	18			33	100
6–10	179		53	87	100
11–15	186			82	100
Adults	46			72	100

3. The Suppressive ED_{99+}

For the suppression of malaria among semi-immune people, it is necessary to know the dosage of chloroquine effective in preventing parasitemia and the intervals of time at which these doses should be administered. It is obvious that the therapeutic ED_{99+} described earlier in this report will suffice for suppression. In previous trials[4] it appeared that an even lower dosage would be enough, provided that any existing parasitemia was cleared at the beginning of the trial. In Table 3 are recorded the results of administration, at 2-week intervals, of various doses of chloroquine, following initial parasite clearance, to semi-immunes of all ages living in an area of holoendemicity. This trial continued for 12 weeks.

The suppressive ED_{99+} shown in Table 3 is, for infants less than 1 year old, 19 mg; children 1 to 2 years old, 37 mg; and all older children and adults, 75 mg.

It must be determined whether or not the suppressive ED_{99+} acts for as long a time as do larger doses. This was done by terminating suppressive treatment of semi-immune children and following them in order to establish the time of reappearance of parasitemia. As has already

been explained, the people are naturally exposed to one infective bite a night and do nothing to protect themselves. This results in trophozoites appearing immediately after the chloroquine level becomes insufficient to suppress them.

Seventy-two children 6 to 15 years old had received 75 mg base chloroquine at intervals of 2 weeks over a period of 12 weeks. After the last dose, 17 days elapsed before trophozoites of *P. falciparum*, the first to return, reappeared in the blood. Among 26 children receiving 120 mg, the period was also 17 days; and among 27 receiving suppressive treatment on the scale 37 to 300 mg, it was 18 days.

CONCLUSIONS

The work described in this report was designed to ascertain the smallest oral doses of chloroquine effective in the treatment and suppression of malaria among semi-immune natives of East Africa. Effective treatment of malaria, irrespective of the species involved, may be obtained with 120 mg base chloroquine, and this dose appears also to provide radical cure of subtertian malaria in people with so advanced an immunity. Less is required for infants and toddlers. The parasite clearance time and relief of symptoms are as rapid with this as with any larger doses, while vomiting and consequent loss of the medicine is more common with the larger doses.

For suppression, 75 mg base administered once every 2 weeks is sufficient, less being needed for infants. A preliminary clearance dose of 120 mg however is essential.

In conclusion, it is interesting to refer again to the work of Stephens and Christophers 60 years ago.[1] The therapeutic ED_{99+} of chloroquine, 120 mg, may be compared with the quantity of quinine required to obtain a 14-day parasite remission (Class III effect of Shannon). Among nonimmunes, the effect is obtained by treatment with 650 mg chloroquine or 3,600 mg quinine, the ratio being 1:5.5.[5] And 120 mg of chloroquine among semi-immunes is, according to this ratio, equivalent to 660 mg of quinine—the dose mentioned by Stephens and Christophers in 1900.

SUMMARY

For semi-immune Africans reared in districts of malarial holoendemicity in East Africa, the following single doses of chloroquine phosphate (recorded as base) are effective in the treatment of malaria caused by any species of parasite and appear to achieve radical cure of subtertian malaria: age 0 to 1 year, 19 mg; 1 to 2 years, 37 mg; 3 to 5 years, 75 mg; and 6 years and older, 120 mg. For suppression, the respective dosages are 19 mg, 37 mg, 75 mg, and 75 mg.

The doses used for treatment clear parasitemia, fever and symptoms attributable to malaria as quickly as do larger doses and stimulate less vomiting. They should precede courses of suppressive treatment.

ACKNOWLEDGMENT

Expectoration of bitter chloroquine by infants was foiled in this investigation by the use of sugar-coated granules given with jam, and I am indebted to Dr. Karl Koenig of the Bayer Company, Leverkusen, for provision of this preparation. This paper is published with the permission of the Permanent Secretary, Ministry of Health, Tanganyika.

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OBSERVATIONS ON TWO *PLASMODIUM FALCIPARUM* INFECTIONS WITH AN ABNORMAL RESPONSE TO CHLOROQUINE

DONALD V. MOORE* AND JACK E. LANIER†

It is generally considered that a radical cure of *Plasmodium falciparum* infections can be effected by the administration of 1.5 grams of chloroquine diphosphate. Several investigators cited by Jeffery *et al.*[1] have advocated the use of single doses of 0.5 and 0.6 g for the elimination of *P. falciparum* infections. Jeffery *et al.*[1] studied over 100 cases of *P. falciparum* in neurosyphilitics using 1.5 g of chloroquine orally and 0.4 g intramuscularly and observed a rapid clinical response as well as a reduction of asexual parasitemias on both regimes. On observing relapses in 3 of 18 patients treated with 0.4 g intramuscularly and a single relapse following a presumed oral dose of 1.5 g, they concluded that the administration of 1.5 g of chloroquine assures elimination of infection. In view of the fact that 3 of 18 cases treated with 0.4 g relapsed they could not recommend this low dose of chloroquine for the termination of *P. falciparum* infections. They had no explanation for the one relapse following treatment with 1.5 g but they recognized the possibility that the patient may not have received the total amount of the drug. Because of the rapid reduction of parasitemia in this case the authors felt that the patient received at least the first dose (0.6 g) and probably the whole 1st-day dose (0.9 g). Assuming that this patient failed to receive the total dose of 1.5 g they point out the potential hazard associated with administration of lower doses of chloroquine.

Recently we observed two patients infected with *P. falciparum* who did not respond to chloroquine in the usual manner. Both patients, employees of a geophysical company, were working on seismographic explorations in Colombia, South America. According to the first patient, he acquired his infection near Sabana de Torres. The second patient did not work in this specific camp but was working in the general area in the Magdalina Valley. In each case information relative to the course of the infection and the drugs administered in South America was obtained from the patient.

The first patient, a 26-year-old white male, could not remember the exact date of onset; but about July 20, 1959, his illness was sufficiently severe to require his transfer to Bogotá where he was hospitalized and the diagnosis of infection with *P. falciparum* was established. At this time he received concurrently injections of chloroquine over a 5-day period and tablets of chloroquine administered over a 3-day period. Definite clinical improvement was observed and the patient was discharged. Approximately 11 days after the above therapy there was a recurrence of malaria paroxysms accompanied by jaundice. The patient returned to Bogotá and was treated with 20 tablets of chloroquine. Approximately 10 days after this course of therapy another relapse was experienced. At this time he was given intramuscular injections of chloroquine as well as tablets of the same drug and sent to the United States.

The patient arrived in Dallas, Texas, on September 4, 1959, and was hospitalized immediately. The diagnosis of infection with *P. falciparum* was confirmed by our laboratory at this time. A complete physical examination was negative except for the presence of a palpably enlarged spleen. The liver was not palpable. There was no jaundice. Liver function tests were normal; stool examination revealed no ova nor parasites. Antimalarial therapy was begun September 5 with the administration of 0.9 g of chloroquine diphosphate followed by 0.3 g on the next 2 days making a total of 1.5 g. Therapy with primaquine was begun September 6 (0.015 g daily for 14 days). The patient was discharged from the hospital September 8 and was seen by J.E.L. September 10. Clinical response to antimalarial therapy was apparent and blood smears for malaria were negative.

On September 19, while still on primaquine therapy, the patient experienced chills and fever. When he was seen September 20 blood smears were taken and chloroquine therapy reinstituted before the blood smears were examined. Examina-

* Department of Microbiology, University of Texas Southwestern Medical School, Dallas, Texas.

† Launey-Altick Medical and Surgical Clinic, Dallas, Texas.

tion of the blood smears confirmed the clinical diagnosis of the recurrence of falciparum malaria.

In view of the fact that the administration of 1.5 g of chloroquine had not effected a cure and that the patient had previously received chloroquine therapy in South America without eliminating the infection, we consulted Dr. Robert Coatney of the National Institutes of Health. The possibility that the patient was not taking the drug seemed to be the only logical explanation for the recurrence of symptoms and parasitemia. It was decided to administer a full course of chloroquine (1.5 g) under strict supervision and in addition determine if chloroquine was being absorbed and excreted in the urine according to the technique described by Haskins.[2] Accordingly a new chloroquine regime was instituted September 22 with the drug being administered under the personal supervision of J.E.L. The following day the Haskins' test for chloroquine was strongly positive. Examination of thick and thin blood films at this time revealed a decrease in parasitemia when compared with the smears made the day before. The chloroquine therapy administered under supervision was completed September 24. On September 28 the Haskins' test was still strongly positive and the thick blood film revealed 5 to 6 ring forms.

Blood smears made October 1 showed an increase in the number of parasites. The Haskins' test was still definitely positive. The night of October 2 the patient experienced a malaria paroxysm with nausea, vomiting, chills, and fever. This paroxysm occurred 8 days after completion of the chloroquine therapy administered under supervision. When the patient was examined the morning of October 3 his temperature was 101.5°F, and the spleen was slightly enlarged and exquisitely tender. Blood smears showed numerous ring forms and a few gametocytes. It is of interest to note that this was the first time gametocytes were observed by us in this patient. The Haskins' test showed that the patient was still excreting chloroquine in the urine. Blood was drawn at this time and sent to Dr. Martin Young, National Institutes of Health, for additional studies on this particular strain of *P. falciparum*. Unfortunately the parasites did not survive.

The patient was started on another therapeutic regime of chloroquine (1.5 g). Clinical response was not as rapid as had been observed previously. Mild paroxysms occurred the next 2 nights and marked clinical improvement was not seen until October 6, the day after the chloroquine therapy was completed. At this time blood was drawn and sent to Dr. Coatney for a chloroquine plasma level determination, which showed a level of 274 μg/L. This is within the upper limits of the expected findings.

Following the completion of 1.5 g of chloroquine the patient was maintained on 0.3 g of chloroquine daily until October 20. Periodic examination of blood smears during the period of October 6 through October 16 revealed a persistence of ring forms. In view of the fact that parasitemia was demonstrable after 14 consecutive days of chloroquine therapy, it was decided to treat the patient with quinine sulphate, 10 grains tid $\times$ 3 and to continue the administration of 0.3 g of chloroquine daily. The quinine therapy was begun October 17 and completed October 19. Blood smears made October 20 revealed no malaria parasites and all antimalarial therapy was withdrawn. One gametocyte was seen in smears made October 22 but no significance was attached to this finding in view of the known persistence of gametocytes after therapy. Blood smears were examined on a biweekly basis from October 22 through November 3 and remained negative during this period.

On November 6 numerous ring forms were seen in thick and thin blood films. This recurrence of parasitemia appeared 17 days after the termination of the quinine therapy, the longest period of time between relapses observed in this patient. Accordingly the patient was retreated with quinine sulphate, 10 grains tid for 10 days beginning November 6. Blood smears on November 9 still contained ring forms, but there had been no clinical relapse. Blood smears were negative November 13 and remained so until the patient was released December 7, 1959.

The second patient, a 34-year-old white male, experienced his primary attack of malaria about the middle of May, 1960. He was taken to Bogotá where he was told that he had two types of malaria. According to the patient he was treated primarily with chloroquine, but he had no information as to dosage or whether other antimalarial drugs were used. His acute illness lasted about 2 weeks. He returned to the United States June 29, 1960.

On July 2, 1960, he again developed chills and fever accompanied by backache and headache. On July 3 and 4 he experienced similar episodes

and on July 5 two such episodes occurred. He was seen by us on July 6. Physical examination was essentially negative except for a slightly enlarged tender spleen, the edge of the spleen being about 2 finger breadths below the left costal margin. Examination of thick and thin blood films revealed numerous ring forms of *P. falciparum*. The patient was hospitalized and a treatment schedule using chloroquine diphosphate instituted. During the hospital stay 2.1 g of chloroquine was administered. Examination of blood smears on July 11, the day after the last dose of chloroquine was administered, revealed ring forms still present. In view of the fact that the patient had been told in South America that he had two types of malaria he was given primaquine diphosphate (15 mg/day × 14 days). No malaria parasites were seen in blood smears made July 15.

As the patient left Dallas to visit his home, he was not seen again until August 5. At this time he stated that he had not felt well during the interval between July 15 and August 5 and that he had experienced a chill and some fever on July 31. Blood smears made at this time revealed rings and gametocytes of *P. falciparum*. Since there was a recurrence of symptoms on July 31, it is logical to assume that this relapse occurred 21 days after the completion of the chloroquine therapy, if not before. On August 7 a specimen of venous blood containing ring stages and gametocytes was taken and sent to Dr. Young. This strain of *P. falciparum* has been successfully established in another patient and is being studied in Dr. Young's laboratory.

In view of our experience with the first patient and because it was imperative that this patient return to work, he was treated, at this time, with quinine sulphate (30 g/day × 10 days). Blood smears were examined twice a week from August 10 to September 1. No ring stages were seen. Gametocytes continued to be present but the number had decreased considerably when the last examination was made.

DISCUSSION

It is well known that resistance of plasmodia to proguanil and pyrimethamine has been developed experimentally and observed in the field. Schnitzer and Grunberg[3] and Bishop[4] have adequately reviewed and discussed the problem of drug resistance in malaria. In the course of experimental work a number of investigators have attempted to produce resistance to Atabrine® (Bishop and Birkett,[5, 6] Williamson *et al.*,[7] Williamson and Lourie[8] and Schmidt *et al.*[9]), but all attempts have failed. Likewise, unsuccessful attempts have been made to produce resistance to the 4-aminoquinoline compounds, chloroquine and Camoquin® by exposure of infected animals to the drugs for more than a year (Thompson,[10] Schmidt *et al.*[9] and Bishop and McConnachie[11]). Strains which had become proguanil-resistant were just as susceptible to chloroquine as the parent strain. More recently Ray *et al.*[12] obtained a two-fold resistance of *Plasmodium gallinaceum* to chloroquine after treatment for 9 months, and Ramakrishnan *et al.*[13] reported a 200-fold resistance of *Plasmodium berghei* to chloroquine after treatment of mice for 7 months. In view of previously reported failures to produce chloroquine-resistant plasmodia, the latter authors raised the question of the metabolism of chloroquine by mice which might explain their success in producing a chloroquine-resistant strain of *P. berghei*.

Field observations on drug resistance in malaria are more difficult to study because of the many complicating factors to be considered. Bishop[4] presents an excellent review of the problem and cites the observations made by numerous investigators in different parts of the world. Schneider *et al.*[14, 15] working in northern Cameroons studied the combined effect of pyrimethamine and chloroquine on *P. falciparum*. They observed the development of resistance to pyrimethamine after several months but did not observe resistance to chloroquine. This is in agreement with other reports concerning the observation of the development of proguanil-resistant strains of *P. falciparum* which exhibited cross resistance to pyrimethamine but remained susceptible to chloroquine.

The *P. falciparum* infections reported here were acquired in Colombia, South America, and both patients failed to respond in the usual manner to chloroquine therapy administered there. Although specific information on the amount of therapy received in South America was not obtained, there is no question as to the adequacy of therapy during this time. The first patient was followed by us from September to December, 1959; during this time he received a total of 10.2 g of chloroquine diphosphate, which is nearly 7 times the usual curative dose of this drug for *P.*

falciparum, and experienced a total of 5 relapses. At one time the parasitemia was observed to persist for 14 days during which period the patient was receiving daily doses of chloroquine. One clinical and parasitic relapse occurred toward the end of a 14-day course of primaquine. Quinine sulphate administered at the rate of 30 grains per day for 3 days produced negative blood smears; however, a parasitic relapse was observed 17 days later. Quinine sulphate was then administered (30 grains a day) for 10 days and apparently produced a cure in this patient. The second patient experienced a clinical and parasitic relapse after receiving therapy in South America and had a recurrence of parasitemia accompanied by minor symptoms after being treated by us with 2.1 g of chloroquine. As in the first case a 10-day course of quinine produced an apparent cure.

An important consideration in determining the drug resistance of plasmodia in patients is the administration of the drug, since failure to swallow the drug is not an uncommon occurrence. We feel that in the cases reported here this can be ruled out as an explanation for the lack of response to chloroquine. The patients were very co-operative and in the first case one course of chloroquine was administered under strict supervision. In addition the Haskins' test demonstrated that chloroquine was ingested and subsequently excreted. This patient developed a chloroquine plasma level of 274 μg/L, which is well within the upper limits of plasma levels observed in patients successfully treated with this drug. The chloroquine therapy of the second patient was administered under hospital conditions.

On the basis of these observations it is impossible to conclude that the experiences reported here represent a strain of *P. falciparum* which is resistant to chloroquine; yet this explanation must be seriously considered. Final conclusions must await other reports of a similar nature or conclusive evidence of the existence of a chloroquine-resistant human plasmodium.

SUMMARY

The first of two patients with *Plasmodium falciparum* infections which failed to respond to chloroquine therapy administered in Colombia, South America, received three further courses of 1.5 g of chloroquine and experienced clinical and parasitic relapses 12 and 8 days, respectively, after the first two courses. Upon the completion of the third course the drug was continued for 14 more days at a dosage of 0.3 g daily. Blood smears remained positive during this period. A 3-day course of quinine sulphate (90 grains) produced negative blood smears, but a parasitic relapse occurred after 17 days. An apparent cure was effected by a 10-day course of quinine.

The second patient experienced a clinical and parasitic relapse approximately 20 days after a first course of chloroquine; and after a second course (2.1 g) followed by a 14-day course of primaquine, a recurrence of parasitemia accompanied by minor symptoms approximately 21 days after the completion of the chloroquine therapy was observed. A 10-day course of quinine produced an apparent cure.

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THE EFFECT OF ERYTHROCYTES ON THE PROPAGATION OF *ENTAMOEBA HISTOLYTICA* IN CULTURE

I. Demonstration of Toxicity of Washed Erythrocytes to a Strain of *E. histolytica**

JAMES G. SHAFFER† and VICHAZELHU IRALU

Department of Microbiology and Public Health, The Chicago Medical School, Chicago 12, Illinois

Phagocytosis of human erythrocytes by *Entamoeba histolytica* was first observed by Lösch[1] in 1873. He described the etiological agent of amebic dysentery and included the statement that red blood corpuscles were found in his "*Amoeba coli.*" Later workers came to regard the ingestion of erythrocytes as a distinguishing characteristic of *E. histolytica*. However, Dobell[2] showed that certain strains of *Entamoeba coli* ingest erythrocytes and warned that errors in diagnosis can occur in this way. *Dientamoeba fragilis*[3] and *Trichomonas vaginalis*[4] have also been shown to ingest red blood cells. According to Kudo,[5] *Trichomonas hominis* and *Balantidium coli* also ingest erythrocytes. None of these other protozoa, however, engulf red blood cells with the same avidity as does *E. histolytica*. Under certain conditions individual trophozoites of *E. histolytica* have been seen to ingest up to 35 red blood cells at a time, in vitro.[6] Dobell[7] reported that the ability to ingest red cells varies from time to time, and that it could even disappear altogether with the passage of time. Animal passage restored phagocytic ability. Shaffer and Balsam[8] defined the "phagocytic index" as the average number of erythrocytes found in 25 trophozoites after a 15-minute exposure at 37°C in the S-F medium and showed that this index is relatively constant for each given strain under standard conditions. Lowered phagocytic indices were demonstrated for amebae treated with specific antisera, or certain drugs. Phagocytosis is said to occur only in acid media (pH 5.6–6.5).[9] Shaffer and Ansfield[6] found lowered phagocytic activity above pH 6.9 and below pH 6.0.

In addition to the phagocytosis of erythrocytes, the ingestion of other particles has been described, including rice starch granules, bacteria and protozoa. When such ingestion occurs, one may ask whether or not such particles are providing necessary nutrients for the amebae. In all probability, the ingestion is a physico-chemical process which may have one of three effects: 1) the particle may contain nutrients which the ameba can use in its metabolic processes and which may stimulate propagation; 2) the particle may be useless to the ameba; or 3) the particle may be toxic to the ameba. It is, of course, possible that a particle might have both growth-stimulating and toxic materials. The outcome of the ingestion of such a particle would depend on the relative concentrations of the materials and their relative stability.

Since the red blood cell (RBC) is so readily ingested by *E. histolytica* and since it is a rather complex particle, it seemed of interest to study the effect of addition of RBC to cultures of the amebae. It was of considerable interest to find evidence, in the experiments to be described, that there exist in the RBC both toxic and stimulatory substances. The findings of these studies are described in this and a succeeding paper.

MATERIALS AND METHODS

At the outset, the K-9‡ strain of *Entamoeba histolytica* and the gram-negative anaerobic bacillus (*Bacteroides*) of Shaffer and Frye[10] were used in the recently devised CLG medium of McDade and Shaffer.[11] Later, experiments were done with the 103 and 200 strains§ of *E. histo-*

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† Present address: The Lutheran General Hospital, 1775 Dempster Street, Park Ridge, Illinois.

‡ The K-9 strain was obtained from Dr. M. M. Brooke of the Communicable Disease Center, Public Health Service. It was brought from Korea in 1951 by Dr. W. W. Frye.

§ The 103 and 200 strains were obtained from Mr. M. C. McCowen of the Lilly Research Laboratories. He obtained them from Dr. C. W. Rees of the National Institutes of Health.

lytica. The method of culture was as follows: 40-ml amounts of the CLG medium in 20 cm × 25 mm test tubes were inoculated with 0.3 ml each of a 48-hour culture of the *Bacteroides* and incubated for 24 hours at 37°C. This was transferred to 40-ml centrifuge tubes, sealed with melted sterile petrolatum and centrifuged at 2,000 rpm for 45 minutes. The supernatant fluid, containing between 90 and 140 million viable cells per ml, was the base medium. After 2.5 ml of the above supernatant fluid was added to test tubes containing 0.8 ml of a mixture of normal horse serum (0.1 ml) and 1,000 units of Penicillin G in 0.7 ml 0.85% NaCl, each was inoculated with 0.5 ml of a suspension of a 48- to 72-hour culture of *E. histolytica*, sealed with petrolatum and incubated at 37°C. Other test preparations were added to the standard 3.8-ml culture.

RBC suspensions were prepared as follows: Blood was collected by cardiac puncture from rabbits and defibrinated. It was centrifuged for 15 minutes at 3,000 rpm to sediment the cells. The cells were then washed 4 times with normal saline. Finally, the washed RBC's were resuspended in saline and centrifuged for 10 minutes at 1,200 rpm. The resulting sediment was defined as a standard 100% erythrocyte concentration. A 5% concentration was made by adding 1 volume of 100% erythrocytes to 19 volumes of saline (0.85% NaCl). To minimize inclusion of white blood cells, sediment was withdrawn from near the bottom of the tube. It was found that, for the purpose of these tests, the whole red cells did not deteriorate up to 1 week when kept in the refrigerator (4°C).

The amebae were counted in a standard spinal fluid counting chamber. Unless otherwise stated, the average number of amebae in eight chambers from two test-tube cultures constitutes each reading. This result was used to calculate either the number of amebae per milliliter or the total number of amebae per tube. When direct examination of the cultures was made, it was done by inverting the test tubes and placing them directly above the condenser of a monocular microscope under low power magnification.

EXPERIMENTAL RESULTS

Action of RBC on E. histolytica *in culture.* A preliminary experiment was done by adding various amounts of a 10% whole defibrinated rabbit blood, ranging from 0.02 ml to 0.54 ml, to standard cultures of the K-9 strain of *E. histolytica*. Direct examination of these cultures indicated inhibition of propagation of the amebae in those cultures with larger amounts of blood. In the control culture tubes growth was normal. The following experiment was then performed to see whether this inhibition might be due to the presence of the RBC. Two sets of cultures were made, (1) routine cultures to which was added 0.1 ml of 5% washed RBC and (2) routine cultures without RBC. Both sets were inoculated with a standard inoculum of 5,469 amebae per tube. The results at 24, 48 and 168 hours are shown in Table 1. At 24 hours, the control cultures were normal in all respects, but examination of the erythrocyte-treated tubes revealed a striking inhibition. The amebae were less active and the number present was actually less than that in the original inoculum. Counts of the control tubes at 48 hours gave a yield of 41,250 per tube, an 8-fold increase from 5,469 originally seeded. Counts were not done on the experimental tubes at this time in this preliminary experiment. The remaining cultures were kept at 37°C and examined on the 7th day of incubation. It was surprising to find a very luxurious growth of amebae in the erythrocyte-treated tubes, previously found to be inhibited on the 1st and 2nd days. At this time the populations in the control tubes had fallen to the usual few survivors. Both the buffy coat over the red cell washings and fresh undiluted serum were tested for their possible effect on the amebae. These were without effect in amounts similar to those used with the RBC. It should be noted at

TABLE 1

The effect of red blood cells on the propagation of the K-9 strain of E. histolytica *over an extended time*

Type of culture	Number of amebae per culture			
	0 hr	24 hr	48 hr	168 hr
No RBC	5,469*	numerous (not counted)	41,250	few alive
0.1 ml 5% RBC added/culture	5,469*	few	none seen	40,000

* Inoculum.

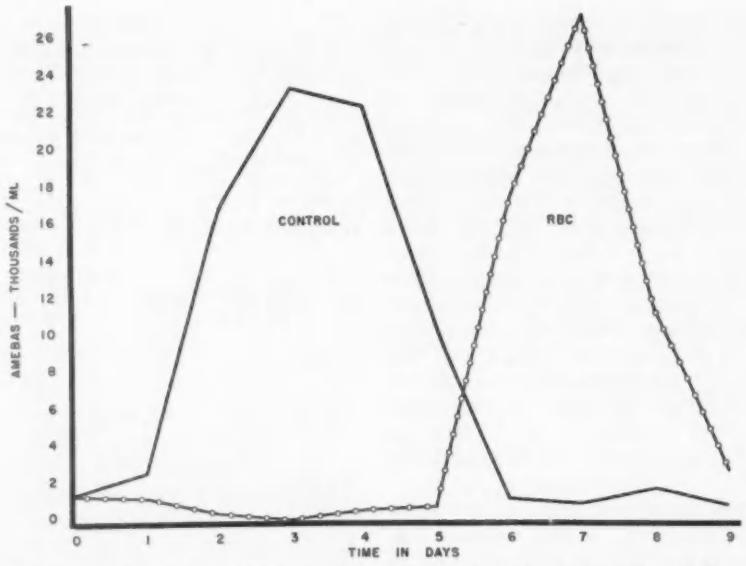

FIG. 1. Growth curves of the K-9 strain of *Entamoeba histolytica* in CLG medium with and without erythrocytes. Each point on the curves was determined by counting duplicate cultures.

this point that, when the routine S-F medium was used instead of the CLG medium, the results were similar.

A comparison of the growth curves of the K-9 strain of E. histolytica *in CLG medium with and without erythrocytes.* In the previous experiments it was obvious that in the erythrocyte-treated cultures a few trophozoites resisted the deleterious effects of the RBC and after the lapse of approximately 5 days the amebae were able to multiply as well as, or better than, those in the control tubes. In order to better characterize the nature of this inhibition, growth curves of amebae under the influence of RBC were determined and compared with the normal growth curve in the absence of RBC.

For these experiments 0.1 ml of a 5% suspension of washed rabbit RBC was used. Sufficient experimental and control cultures were inoculated to provide duplicate tubes for counting at appropriate intervals. The pH of the completed culture tubes just prior to sealing was 6.0. This did not vary appreciably during the course of the experiment.

The results of this experiment are shown in Figure 1. CLG cultures without the erythrocytes showed a standard growth curve comparable to that obtained by McDade and Shaffer.[11] The peak of the growth curve was reached on the 3rd day (72 hours) after which the death phase began. By the 6th day few amebae remained.

In contrast to the control, erythrocyte-treated cultures showed a marked decline in population by the 2nd day (48 hours), with a further decrease on the 3rd day. This suppression of population was maintained for 5 days. On the 6th day a marked increase occurred, and by the 7th day there was an 18-fold increase, which exceeded the peak of the control cultures. After this, the population declined and by the 10th day returned to a figure comparable to the original inoculum.

TABLE 2

The toxicity of red blood cells for the K-9 strain of E. histolytica *in CLG medium*

Washed red blood cells (5%)	Number of amebae per culture (3.8 ml)*	
	0 hr (inoculum)	72 hr
None (control)	6,250	47,500
0.1 ml	6,250	1,330
	12,500	1,520

* Each determination represents an average of 2 cultures.

Demonstration of the toxicity of red blood cells on the K-9 strain of E. histolytica. In the previous experiments, it appeared that the population actually declined under the influence of the RBC. In order to determine the possible extent to which the amebae were being destroyed, a much larger inoculum of amebae was used in the next experiment. The cultures were examined 72 hours after the start of the experiment, a time at which in the previous experiment the lowest counts had occurred. Table 2 shows counts made after 72 hours from cultures originally inoculated with 6,250 and 12,500 amebae together with RBC, and with 6,250 amebae and no RBC as a control. The amebae in the control increased from 6,250 to 47,500 whereas the red cell treatment reduced the original 6,250 to 1,330 and 12,500 to 1,520. Thus, only 21% and 12% of the original inocula survived after 72 hours. Figures 2 and 3 show typical CLG cultures of the K-9 strain with RBC, after 72 hours. Trophozoites in progressive stages of intoxication and death can be seen. Some are without any ingested RBC, whereas others have various numbers of intact RBC or degenerate red-brown masses. Some brown pigment granules can be seen lying free in the medium and are presumably detached from trophozoites which had once harbored them. These combined observations,

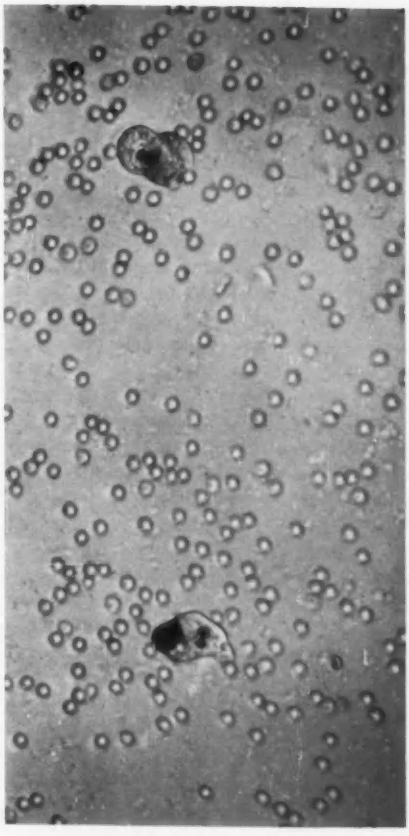

FIG. 2. Typical 72-hour CLG culture of the K-9 strain of *Entamoeba histolytica* with erythrocytes. Trophozoite below with partially digested RBC. Erythrocyte-free ameba above.

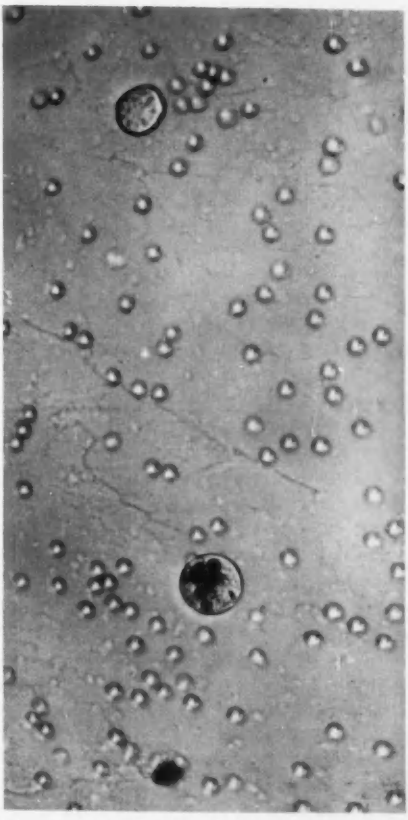

FIG. 3. Typical 72-hour CLG culture of the K-9 strain of *Entamoeba histolytica* with erythrocytes. Freshly ingested and partially digested RBC seen inside ameba. Note the pigmented clump lying free in the medium, at bottom. This may represent what remains after a trophozoite has degenerated.

i.e., the actual decrease in absolute numbers of amebae, and the suggestive brown pigments, in the older culture fluids as well as in the bodies of the trophozoites, would suggest that red cells are directly toxic to the trophozoites in some way. In other experiments, as little as 0.1 ml of a 0.1% RBC suspension produced very marked depression of amebic growth in standard CLG cultures.

The apparent resistance of certain strains of amebae to the action of red cells. In addition to the K-9 strain tested thus far, two other large race *E. histolytica* strains, 103 and 200, were tested with the standardized 0.1 ml of 5% washed RBC. Although their growth appeared to be slightly inhibited by the red cells during the first 2 days as compared with the controls, by the 3rd day there was little difference between experimental and control cultures. This was quite different from observations on the K-9 strain. With the 103 and 200 strains the red cells began to lyse after 48 hours in the cultures. As a result of this observation, it was important to determine whether or not this lysis was caused by the amebae or by some bacterial contaminant that was present in the 200 or 103 cultures. Bacterial subcultures failed to reveal the presence of any bacterial contaminant, but since there might be such an organism present which would not grow out readily on subculture, another experiment was done. Portions of the fluid above the amebae were transferred to fresh tubes of the CLG medium, with care being taken to avoid picking up amebae. After this, other tubes were inoculated with amebae from the same 48-hour cultures. RBC were then added to all tubes and incubated at 37°C. Lysis of the RBC occurred within 48 hours in all tubes containing propagating amebae, but not in tubes inoculated with the culture fluid. These results suggest strongly that the lysis of the RBC was produced by the amebae and not by any bacterial contaminant which might be present in the cultures.

The toxicity of the red cells of other vertebrates. So far the RBC of 6 different rabbits have been used and all have exhibited the same effects. The RBC of several other vertebrates have been tested for this property of toxicity. The RBC of a number of humans, dogs, guinea pigs, mice, and turtles have been tested. All, except those of the turtle, showed inhibitory power, comparable to that of rabbit cells, on the growth of the K-9 strain of *E. histolytica*. The degree of inhibition by the large, oval, nucleated RBC of the turtle (*Chrysemys picta*) appears to be considerably less than that of the rest, possibly because phagocytosis of a large number of these cells by the amebae is not easy. So far, the RBC of these species, other than the rabbit, have not been checked with strains 200 and 103.

DISCUSSION

E. histolytica perhaps takes in some nutrient materials from its surroundings by means of simple osmosis, and by "drinking" liquid material (pinocytosis), as has been shown for a few free-living amebae.[12, 13] But by far the most obvious and easily observed activity that may be important in the nutrition of this protozoan is its phagocytosis. *E. histolytica* readily ingests numerous types of inanimate and animate particles as indicated previously. On the other hand, it rejects such particles as charcoal, Fuller's earth, filter-cel, celluflour, and barium sulfate,[14] thus showing a certain amount of selectivity.

As has been pointed out earlier, a single trophozoite can engulf up to 35 erythrocytes. Considering that a single trophozoite may measure 20 to 25 μ in diameter and a red cell about 7 μ, the ability to ingest so large a number at one time is remarkable. Since red cells are so richly endowed with a number of simple and complex physiological substances including enzymes active in normal metabolic processes, it is surprising to find that not much investigation has been carried out on this material in the study of the biology of this parasite. Anderson *et al.*[14] rightly observed thus: "In the metabolism of the amebae, perhaps too little attention has been paid to erythrocytes which are readily ingested by cultured trophozoites."

Many parasites live in close association with blood. Members of the genus *Plasmodium* actually live inside the red cells of different vertebrates during parts of their life cycle. In laboratory cultures, blood is frequently employed as a supplement necessary for successful growth of microorganisms. Certain bacteria, such as *Hemophilus*, require certain erythrocytic materials for growth. The need of the trypanosomes for red blood cells is well known.

This series of experiments indicates that under certain conditions, erythrocytes may be detrimental to the growth and multiplication of the trophozoites of *E. histolytica*. Since fresh serum, constituting almost half the volume of whole

blood, can also kill and even lyse the trophozoites of *E. histolytica*[15-17] this had to be ruled out as a possible source of toxicity. So were the white blood cells which have certain well-known antimicrobial effects. Inhibitory power was found to be due primarily to the red cells and not to the serum or white blood cells in the amounts used; and since most of the amebae in the cultures ingested RBC, it is reasonable to suggest that the ingested RBC was the toxic agent.

The ability of strains 103 and 200 to grow in the presence of RBC may be due, at least in part, to one or both of the following observed characteristics. Both strains hemolyze the RBC, an ability which may prove to be of considerable importance. The two strains also migrate up the walls of the culture tubes very extensively. K-9 trophozoites remain at the bottom of the tubes and are not hemolytic. It would seem likely that the ability of strains 200 and 103 to lyse the RBC is of greatest importance, since this would result in dilution of the toxic materials. Whether this hemolytic substance is produced extracellularly or is related to what Craig[18] described as an essentially intracellular material is not clear at present.

Several very interesting questions are raised by this phenomenon of inhibition and recovery of amebae under the influence of erythrocytes. First of all, one of the most obvious questions is the identity and mechanism of killing of the substance or substances involved. Another paper deals with the identification of substances present in erythrocytes which appear to be responsible for the killing of the amebae. The suppression of the population with strain K-9 lasts for approximately 5 days, after which the amebae grow out. Present evidence indicates that a considerable amount of hemolysis occurs by the 5th day and this may mean that the toxic substance becomes diffused in the medium. Hemolysis in the K-9 cultures appears to be associated with aging of the RBC, not with any specific effect of the amebae. The toxic material present in whole RBC may constitute concentrated packages of the substance(s) responsible for killing, whereas hemolysis might cause this material to become diluted, and much less available.

Still another question can be asked about the amebae that survive the killing action of erythrocytes. Two explanations may be offered. According to unpublished data obtained in this laboratory, it appears that the phagocytic index varies with the physiological age of the amebae; rapidly dividing cells have a low index, whereas resting cells phagocytize more red cells. In any given population of amebae a few do not phagocytize red cells, or if they do, only a few red cells are taken in, even though large numbers are available to them. These amebae presumably might survive and multiply at a later time. The other explanation rests on the manner in which the red cells settle down in relation to the bulk of the amebic population in a culture. Almost all the suspended red cells settle to the bottom of the culture tube, forming a button of packed red cells. Most of the K-9 amebae also settle to the bottom. A few, however, adhere to the sides of the test tubes from the beginning or crawl up from the bottom. In this way a few probably fail to ingest RBC.

Finally, the toxicity of the erythrocytes to the amebae is rather paradoxical because these organisms apparently readily ingest this material in nature. While the possibility exists that red cells have certain potent food value to the amebae, they also contain a chemical entity or entities detrimental to them. What role this plays in the economy of nature can be only vaguely guessed. One can speculate that this is yet another mechanism of natural defense possessed by the body of the vertebrate host against certain strains of this parasite. One may also speculate as to whether or not the effect of strains 200 and 103 on the RBC is in any way related to pathogenicity. In addition to the strongly amebicidal and amebilytic powers of fresh normal serum, one can visualize that red cells form yet another natural defense barrier against certain of the amebae in the tissues, particularly in the vascular system.

SUMMARY

It has been found that the addition of red cells to the CLG and S-F media inhibits the multiplication of the K-9 strain of *Entamoeba histolytica*. After about 5 days the amebae recover from the inhibitory action of the erythrocytes and grow in a normal fashion when optimum amounts of red cells and amebae are used. During the inhibitory period there is actual destruction of many of the trophozoites originally seeded, suggesting the presence of a toxic material in red cells. *E. histolytica* strains 103 and 200 showed resistance to the effect of red cells. It was observed that these strains readily hemolyze red

cells. This, and their ability to migrate up the sides of the culture tubes, may explain their resistance to the red cells' effects which were observed with the K-9 strain.

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THE EFFECT OF ERYTHROCYTES ON THE PROPAGATION OF *ENTAMOEBA HISTOLYTICA* IN CULTURE

II. The Effect of Red Blood Cell Derivatives*

VICHAZELHU IRALU and JAMES G. SHAFFER†

Department of Microbiology and Public Health, The Chicago Medical School, Chicago 12, Illinois

In a previous paper it was demonstrated that whole red cells of certain vertebrates exerted an inhibitory action on the trophozoites of the K-9 strain of *Entamoeba histolytica* in culture.[1] This effect was not seen when the 103 and 200 strains of *E. histolytica* were used. The literature dealing with the antimicrobial properties present in the erythrocyte and its products is rather scanty. Reference to antiprotozoal action, if any, has not been found. One of the earliest references on this general subject is by Kämmerer[2] who claimed that mesohematin inhibited certain bacteria. Hematin was found to be antibacterial by van Heyningen.[3] Waksman[4] listed the red cell product "erythrin" as an antibiotic substance. Whitney *et al.*[5] reported that hydrolyzed products of erythrocytes were antibacterial and they attributed this activity to some peptide amino acid complexes. Hobson and Hirsch[6] demonstrated that the antibacterial activity of hemoglobin is due to the globin moiety of the molecule. Wood and Ono[7,8] claimed that the intact porphyrin in hemoglobin and its derivatives could account for the inhibitory activity. Dubos[9] believed that the porphyrins should have iron in them to exhibit this toxicity.

The present paper deals with the further characterization of the inhibitory substance(s) in erythrocytes which are active against *E. histolytica*.

MATERIALS AND METHODS

The CLG medium was used with the K-9, 103, and 200 strains of *E. histolytica* as described earlier.[1] Rabbit red cells were likewise washed as previously described.[1] The cells were lysed by mixing 1 volume of 100% erythrocyte with 4 volumes of sterile distilled water. Microscopic examination revealed complete lysis of all the erythrocytes. This represented 20% red cells by volume.

Separation of the insoluble stroma from the soluble portion of the lysate was effected by centrifuging lysed cells at 3,000 rpm for 30 minutes. The clear supernatant was removed by a capillary tube and used without further processing. The stroma residue was washed 4 times with about 10 times its volume of saline. At the end of the washing process the supernatant became clear but the stroma mass retained a pink color suggesting incomplete removal of hemoglobin. This was resuspended in saline to correspond to the same volume of lysed material from which it was prepared.

Washed whole cells (100% erythrocyte), lysed cells (20%), supernatant, and washed stroma were lyophilized in shallow cylindrical dishes. Overnight processing of amounts ranging from 5 to 40 ml of the above preparations produced dry products which could be ground to a fine powder. The minimum dry weight yields, in grams, from original preparations of 5 ml of 100% washed cells, 10 ml of lysed cells, 28 ml of supernatant fluid, and the stroma of 40 ml of lysed cells were 1.25, 0.48, 1.38, and 0.32, respectively. These lyophilized materials were weighed and dissolved in either saline or distilled water to make appropriate concentrations.

For the purpose of dialysis, 500 mg of the dried supernatant material was dissolved in 4 ml of distilled water. This solution was placed in a narrow Visking cellophane bag and dialyzed against 4 ml of distilled water in a large screw-capped test tube for 24 hours. At this time the liquid volume inside the bag increased from 4 ml to approximately 5 ml, and the clear liquid outside contracted from 4 ml to about 3 ml.

In addition to the above materials, twice recrystallized bovine hemoglobin, recrystallized hemin, and hematoporphyrin hydrochloride, all

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† Present address: The Lutheran General Hospital, 1775 Dempster Street, Park Ridge, Illinois.

obtained from the Nutritional Biochemical Corporation, Cleveland, Ohio, were used. From 10 to 15% saline solutions of hemoglobin were made. Since hematoporphyrin hydrochloride was but slightly soluble, and hemin was insoluble in saline, it was necessary to add 0.03 to 0.05 ml of 1N NaOH to each milliliter of suspensions containing 5 mg/ml of these compounds. The three solutions were sterilized by passing through sintered glass filters.

EXPERIMENTAL RESULTS

Test for inhibitory power in lysed cells, stroma-free supernatant, and washed stroma preparations on the multiplication of E. histolytica. Standard inocula of amebae were cultured in the presence of 0.01, 0.04, 0.07, and 0.10 ml of lysed cells and stroma. Also, cultures with 0.01 and 0.10 ml of supernatant preparation were made. Each of these preparations corresponds to material present in a 20% saline suspension of RBC. Controls consisted of cultures with and without 0.1 ml of 5% erythrocyte. Table 1 shows the yields after 48 hours of incubation at 37°C. It will be noticed that the lysed cell suspension and the supernatant showed little activity. The stroma exhibited some inhibition, which was probably due to the presence of toxic material not removed by the washing and to the tendency of the stroma to settle out in a manner similar to that of the RBC.

TABLE 1

Comparison of the inhibitory powers of lysed red blood cells, their stroma, and the soluble portion of the red blood cells, on the K-9 strain of E. histolytica

Red cell preparation	Amount (ml)	Amebae per ml (48 hr)
Lysed cells (20%)	0.01	9,296
	0.04	15,312
	0.07	15,859
	0.10	19,609
Stroma, corresponding to 20% lysed cells	0.01	8,984
	0.04	12,031
	0.07	5,703
	0.10	8,359
Soluble portion (supernatant) from 20% lysed cells	0.01	14,375
	0.10	14,375
Controls	No RBC	15,234
	0.1 (5% RBC)	78

Concentration of the inhibitory substance(s) present in the red cell, lysate, stroma-free supernatant, and washed stroma by lyophilization. It was found as reported previously that 0.10 ml of a 5% red cell suspension exerted a marked suppressive effect on standard CLG cultures of strain K-9 *E. histolytica*. In the preceding experiment, however, at the concentrations used, the supernatant and lysed cells were not inhibitory. It was thought that this failure may have been due to dilution of the soluble material. In order to obtain concentrated forms of the preparations, washed whole cells, whole lysate, supernatant and stroma were lyophilized as previously described. The preparations were then accurately weighed and 10% solutions (W/V) prepared in distilled water. To standard CLG cultures of the amebae, the equivalent of 0.5 mg and 2.5 mg of the dried material per milliliter of culture was added. The results, as shown in Table 2, indicate that exposure to CO_2 ice temperature in the lyophilization process did not destroy the inhibitory activity of the preparations. All the preparations, especially at the high concentrations, were markedly toxic. Further experiments showed that about 8 mg/ml of dehydrated supernatant was roughly equivalent to the use of 0.1 ml of a 5% cell suspension (which, expressed in dry weight, is equivalent to about 0.32 mg per ml).

Dialysis of the supernatant. Since the soluble supernatant was found to contain toxic material, it was subjected to further analysis. In order to determine whether the molecules responsible were small and diffusible, the supernatant was dialyzed for 24 hours and the contents inside and outside the bag were tested. A slight departure from the usual procedure of culture was made to allow for the fairly large volumes of dialysate materials added. The usual amount of saline used in the mixture, namely, 0.68 ml per culture, was replaced by 0.68 ml of the dialysate or 0.3 ml of dialysate plus 0.38 ml of saline. It was found that both 0.3 ml and 0.68 ml of the nondiffusible contents of the dialysis bag were highly toxic; whereas the clear material from the outside of the bag showed little activity (see Table 3).

A comparison of the toxicity of commercially prepared bovine hemoglobin and other hemoglobin derivatives to E. histolytica. The preceding experi-

TABLE 2

The effect of adding lyophilized whole red blood cells, lysed cells, soluble portion, and stroma to standard cultures of the K-9 strain of E. histolytica

Material	Milligrams of lyophilized material per ml of culture	Amebae per ml (72 hr)
Whole cells	2.5	3,984
	0.5	10,390
Lysed cells	2.5	5,312
	0.5	8,281
Supernatant	2.5	5,000
	0.5	12,031
Stroma*	2.5	3,359
	0.5	7,343
Control	0	21,718

* Stroma apparently contained hemoglobin and possibly other materials, in spite of repeated washing; thus, the activity shown may be the result of imperfect washing.

TABLE 3

Comparison of the toxicities of the dialysis products of lysed red blood cell supernatant to the K-9 strain of E. histolytica

Source of material	Amount (ml)	Saline added (ml)	Yield of amebae per ml (72 hr)
Inside the dialysis bag	0.30	0.38	625
	0.68	—	156
Outside the dialysis bag	0.30	0.38	8,203
	0.68	—	14,218
Control	—	0.68	11,875

ment indicated that the toxic material was a non-diffusible one. The substance present in the highest concentration in the dialysis bag was, of course, hemoglobin. It was therefore decided to test hemoglobin and its derivatives for possible toxicity to the amebae.

Twice recrystallized bovine hemoglobin, recrystallized hemin and hematoporphyrin hydrochloride were prepared as indicated previously and tested for toxic activity. Hemoglobin was found to be inhibitory at concentrations of 8 mg/ml of culture fluid. Hemin and hematoporphyrin did not appear to be toxic when in suspension; but when these two substances were dissolved in minimum amounts of alkaline solutions, hemin was found to be highly toxic, while the effect of hematoporphyrin was quite different as will be shown below. As little as 0.075 mg/ml of hemin was able to suppress the growth of amebae. As much as 2 mg/ml hematoporphyrin HCl failed to kill. The 103 and 200 ameba strains were killed by hemoglobin and hemin at the same toxic doses as for K-9. Hematoporphyrin did not kill these strains at the high level of concentration.

Stimulatory-inhibitory effects of hemoglobin and some of its derivatives on the multiplication of E. histolytica. In the preceding experiment, whereas milligram amounts of hemoglobin and hemin consistently exhibited toxic action on the amebae, erratic results were obtained from lower levels of concentrations. As a matter of fact, in some instances, better than control yields were obtained. Since certain chemicals are known to have both inhibitory and stimulatory actions on the growth of certain microorganisms, depending on the amounts present, a wide range of concentrations of hemoglobin, hemin, and hematoporphyrin was tested in this experiment.

To standard CLG preparations, varying amounts of filtered concentrated hemoglobin solution were added, so as to produce final concentrations varying from 65 to 7,500 μg/ml of culture. Counts made after 24 hours of incubation at 37°C revealed stimulation of propagation in concentrations from 65 to 1,250 μg/ml. Representative counts were 4,453 amebae per milliliter at 65 μg; 4,800 at 325 μg; and 4,296 at 1,250 μg, as compared to 2,850 amebae per milliliter in the control without hemoglobin. In concentrations from 3,750 to 7,500 μg/ml the yield was almost identical with that of the control. As shown in the previous experiments, concentrations of 8,000 μg and above showed definite toxicity. The reasons for the rather wide range of concentrations which showed equal stimulation are not immediately apparent. It could be that the stimulatory effect is not a matter of supplying a needed nutrient to the amebae. In the 3,750- to 7,500-microgram range it might be that the toxicity and stimulation are balanced, toxicity being more potent than stimulation at concentrations above 7,500 μg. Another point of some interest is that the stimulatory effect of the lower concentrations is more evident after 24 hours

than it is after 48 hours of incubation, whereas the toxic effect of the higher concentrations is more evident after 48 hours.

Hemin was tested in a similar fashion, but with much lower concentrations. The range in this case was from 6 to 75 μg. Again, the lower concentrations showed evidence of stimulatory effects. After 24 hours, cultures containing 6, 12 and 25 μg/ml yielded counts of 4,140, 4,870 and 5,312 amebae per milliliter, respectively, while the control had 3,281 amebae per milliliter. At a concentration of 75 μg/ml the count was 1,328 amebae which indicates definite inhibitory activity. After 48 hours, only the 12-microgram concentration showed evidence of stimulatory activity. Thus, there appears to be a narrow range of concentration of hemin which, in these experiments, showed evidence of stimulatory activity. At 75 μg/ml at 48 hours there were only 2,812 amebae per milliliter, while the control had 13,400 amebae per milliliter. It is not certain whether this effect is amebicidal or amebistatic.

Hematoporphyrin was tested over a range of concentrations from 6 to 2,000 μg/ml. In the low concentrations (6 to 50 μg), results were irregular, indicating no effect. There was evidence of stimulatory effect, most obvious after 48 hours of incubation, in concentrations of 125 and 250 μg/ml. These concentrations yielded counts of 19,609 and 17,656 amebae per milliliter as compared to the control which had 12,656 amebae per milliliter. Increasing the concentration above 250 μg/ml did not result in greater stimulation.

DISCUSSION

It is interesting to note that the ratio of the toxic doses of hemin and hemoglobin is in the proportion of 0.075 mg to 8.0 mg, or roughly 1:100. This happens to be about the same ratio of the molecular weights of these two substances. This suggests the possibility that the toxic property is due to the heme portion in both instances. The activity of the hemoglobin molecule could be, therefore, due to the hemin moiety.

These findings on the antiamebic effects of hemoglobin and its derivatives are similar to those on the antibacterial action of these substances reported in the literature. It is consistent with the findings of Kämmerer,[2] von Heyningen,[3] Dubos,[9] and Wood and Ono.[7, 8] The lack of toxicity of hematoporphyrin is in accord with the observations of Dubos[9] which indicate that iron should be present in the porphyrin to exhibit toxicity. The antibacterial property ascribed to "amino-acid complexes" by Whitney *et al.*[5] and to the globin moiety by Hobson and Hirsch[6] needs further verification.

Although the 103 and 200 strains of *E. histolytica* resisted the effects of whole RBC,[1] apparently by lysing the red cells and/or by migrating up the tube, it appears now that their resistance was only apparent, since they were affected by hemoglobin and hemin in a manner similar to that of strain K-9.

The stimulatory effects of small amounts of hemoglobin, hemin and hematoporphyrin HCl are of interest in the study of the nutrition of this protozoan. Proteins are said to stimulate food intake in certain free-living amebae studied.[10, 11] To what extent hemoglobin, as a protein, stimulated ingestion of food and produced a higher population has not been determined. Hemoglobin is reported to be present in the ciliate *Paramecium* in a concentration of 1.12 to 1.74%, and in *Tetrahymena* in a concentration of 0.22 to 0.3%.[12] Other tetrapyrroles related to the heme are widely distributed in microorganisms.[13, 14] Cytochromes, cytochrome oxidase, catalase, peroxidase, some dehydrogenases and nitrate reductases may also be found. Since *E. histolytica* is an anaerobic organism, it is interesting to note that cytochrome b was reported from *Trichomonas fetus*.[15] Some microorganisms are noted for their inability to synthesize porphyrins. The requirement of hemin, or "X-factor," by *Hemophilus influenzae* is well known. According to Lwoff, no leishmania or trypanosome is known that does not require exogenous hematin.[16]

It is perhaps worthwhile to point out that Cowperthwaite *et al.*[17] used 25 μg of hemin per milliliter in their medium which permitted the growth of *Herpetomonas culicidarum*, the first species of the Trypanosomidae to be cultured in a synthetic medium. Demain and Hendlin[18] found optimum stimulation of *Microbacterium sp.* at concentrations of 50 μg and less of hemoglobin, hemin, and protoporphyrin. The values for stimulation found in the present experiments fall within these ranges.

With the present cultural technics it will be difficult to study the role of these heme compounds in the nutrition of *E. histolytica*. No attempt has yet been made to determine whether these are essential constituents of this parasite, or whether *E. histolytica* can synthesize its own

requirements. Further study of these and related substances would seem to be indicated.

SUMMARY

Upon concentration, hemolyzed red cells and stroma-free supernatant were found to be inhibitory to the K-9 strain of *Entamoeba histolytica*. Dialysis of the concentrated supernatant revealed that the toxicity probably resided in a non-diffusible, high molecular weight substance. Hemoglobin was found to be highly inhibitory to the K-9, 103, and 200 strains at levels of 8 mg/ml; and it is likely that this substance accounts for the toxicity of RBC. Hemin in solution was found to be highly toxic to these three strains at levels of 75 μg/ml of culture. It is suggested that the heme moiety is responsible for the toxicity of both hemoglobin and whole RBC to the ameba. Low concentrations of hemoglobin and hemin showed a stimulatory effect on the multiplication of *E. histolytica*. Hematoporphyrin HCl showed only stimulatory effects. All evidence indicates that the effects of hemoglobin, hemin and hematoporphyrin on strains K-9, 103 and 200 are identical. This would seem to suggest that the resistance of strains 103 and 200 to the effects of whole RBC as noted in another publication was the result of the ability of these strains to cause lysis of the erythrocytes. Such lysis results in considerable dilution of the soluble materials of the RBC.

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THE INCIDENCE OF INTESTINAL PARASITES IN AMEBIC AND BACILLARY DYSENTERY

S. J. POWELL, ESME HENNESSY, A. J. WILMOT AND R. ELSDON-DEW

*The Department of Medicine, University of Natal, The Amoebiasis Research Unit, Durban, South Africa**

Though *Entamoeba histolytica* is the most common animal parasite producing dysentery in man, helminth infection may also play some part. Heavy loads of *Schistosoma mansoni* and *Schistosoma japonicum* are well recognized examples. Massive infection with *Trichuris trichiura* can result in dysentery[1] and this parasite has been recognized as a cause of dysentery in Indian and African children in Durban.[2] It is possible that some of the other intestinal parasites commonly accepted as non-pathogenic may produce similar symptoms but dysentery in adults in Durban is regarded as a rare sequel to infection with such parasites.

It has been estimated that *E. histolytica* inhabits the gut of about 10 per cent of the world's population and yet it produces dysentery in comparatively few. This has been taken to imply that invasion of the gut wall by a usually commensal *E. histolytica* is brought about by some other factor acting as a trigger. Such a factor has not been clearly demonstrated but it has been suggested that associated intestinal parasites may be of importance. In Durban, where severe amebic dysentery is common in the African population, it has been shown that there is a high incidence of other intestinal parasites, notably such helminths as *Trichuris* and *Ascaris*.[3-6] The present work is a study of the relative incidence and severity of infection with these parasites in groups of African patients with dysentery.

MATERIALS AND METHODS

The investigation was planned to provide information on (1) 100 African males with proved acute amebic dysentery; (2) 50 African males with proved bacillary dysentery; (3) a control group of 100 African males with miscellaneous medical, non-dysenteric conditions.

Beaver's[7] technic of counting the eggs in a 1- to 2-mg fecal smear was used, as repeated counts on the same stool specimen in this laboratory had confirmed Beaver's finding of reasonably consistent results. In addition, all the specimens were examined by zinc sulfate flotation to reduce the possibility of missing minimal infections. The presence of all parasites was recorded and the degree of worm infection was assessed as follows on five successive daily stools from each subject: *Trichuris* and hookworm, light, less than 5, and heavy, more than 20 eggs per preparation; *Ascaris*, light, less than 20, and heavy, more than 75 eggs per preparation.

The dysenteric groups received a combination of tetracycline, diiodohydroxyquinoline and chloroquine following the initial stool examination. Those with amebic dysentery were observed for 27 days during which daily stools were examined and sigmoidoscopy performed on the 1st, 5th, 10th, 15th, 20th, and 27th days following admission.

RESULTS

Table 1 shows the distribution of all the parasites found in the three groups. Table 2 indicates the degree and incidence of *Trichuris*, *Ascaris* and hookworm in these groups. Our findings indicate that:

1. The incidence of *Trichuris* (84%) and *Ascaris* (54%) was significantly higher in the patients with amebic dysentery than in either the control group (63% and 40%) or in those with bacillary dysentery (56% and 36%).

2. Despite the higher incidence of certain helminths, the patients with amebic dysentery had a much lower incidence of protozoa other than *E. histolytica*. This was particularly marked with the figures for *Entamoeba coli* (4%), *Endolimax nana* (3%), *Iodamoeba butschlii* (1%) and *Chilomastix mesnili* (4%).

3. In the patients with bacillary dysentery, whereas the incidence of helminths paralleled that in the controls, the over-all incidence of protozoa was significantly lower. Apart from *E.*

* P. O. Box 1035.

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histolytica it was higher than that in the patients with amebic dysentery.

Although treatment produced clinical cure in the group with amebic dysentery as judged by disappearance of symptoms, healing of ulcers and removal of *E. histolytica* from the stools, other protozoa and the helminths were not materially affected apart from a reduction of *Ascaris* incidence to 36%.

DISCUSSION

In his initial investigation of amebiasis in the Durban African, Elsdon-Dew[5] showed that by comparison with other parts of the world the incidence of the commensal protozoa such as *E. coli*, *I. butschlii*, *E. nana*, *C. mesnili* and *Giardia lamblia* was relatively low and the present results confirm this observation with the exception of *G. lamblia*. Our present control series is not strictly parallel with the surveys on apparently healthy Africans done by Elsdon-Dew and Horner[6] as these investigations were done on a single specimen whereas the present investigation covered five specimens. Our control group shows that the incidence of these protozoa in Africans without symptomatic amebiasis resembles that reported in other parts of the world. Possibly the somewhat lower incidence of protozoa in both amebic and bacillary dysentery is due to the bowel disturbances. It is of interest to note that the incidence of *E. histolytica* in our control group was similar to that reported in many places where symptomatic amebiasis is rare.

Elsdon-Dew[4, 6] showed that the incidence of helminths, particularly of *Trichuris* and of *Ascaris*, is high in the Durban African. Our present control group indicates an even higher degree of infection and though there is no significant difference in the incidence of helminths between patients with bacillary dysentery and controls, those with amebic dysentery have a significantly higher incidence of *Trichuris* and *Ascaris*, but not of tapeworm nor of hookworm, than do the controls.

Comparing the incidence of parasitic infection between a slum at Cato Manor and the adjoining Municipal Housing Scheme at Chesterville, Elsdon-Dew[5] has shown that there is a significantly higher incidence of helminths in Cato Manor than in Chesterville, but that of protozoa is the same in the two areas. This may be explained by the fact that protozoa reproduce in the bowel and thus persist regardless of the ex-

TABLE 1

Comparison of intestinal parasites in Africans with amebic dysentery, with bacillary dysentery and without dysentery (in per cent)

	Amebic dysentery	Bacillary dysentery	African controls
Trichuris	84	56	63
Ascaris	54	36	40
Hookworm	20	12	18
Strongyloides	4	4	0
S. mansoni	3	8	2
S. haematobium	3	0	0
Hymenolepis	2	2	0
Taenia sp.	19	24	17
E. histolytica	100	0	11
E. coli	4	26	40
E. nana	3	16	32
I. butschlii	1	4	9
C. mesnili	4	6	14
G. lamblia	8	6	5
T. hominis	14	14	6
Unidentified small amebae	2	4	0
E. hartmanni	1	0	5
Isospora	0	0	2

TABLE 2

Degree and incidence of Trichuris, Ascaris *and hookworm infections (in per cent)*

	Amebic dysentery	Bacillary dysentery	African controls
Trichuris			
Light	65	54	58
Medium	13	2	14
Heavy	6	0	1
Total	84	56	73
Ascaris			
Light	38	30	28
Medium	7	4	9
Heavy	9	2	3
Total	54	36	40
Hookworm			
Light	19	12	18
Medium	1	0	0
Heavy	0	0	0
Total	20	12	18

ternal factors whereas most helminths undergo a cycle outside the body and re-infection is necessary to replace the worms which are expelled. Infection with *Ascaris* and *Trichuris* is due to the ingestion of soil earlier contaminated with fecal material and the high incidence of these worms at Cato Manor probably follows on poor hygiene and lack of sanitation. In contrast to a low incidence at Chesterville, a high proportion of our patients with amebic dysentery come from Cato Manor, suggesting that the poor environmental conditions in this slum are important epidemiological factors in the production of the disease. This positive correlation between the high incidence of *Trichuris* and *Ascaris* and amebic dysentery may be due to a common factor of poor hygiene as the parasites have a similar ecology. However, inadequate sanitation and hygiene can also be related to the incidence of bacillary dysentery and as this group does not show a higher incidence of *Ascaris* and *Trichuris* than do the controls this may indicate a more direct association between the presence of these helminths and the development of acute amebic dysentery.

Though the incidence of helminths in Durban Africans is high, the present investigation indicates that the weight of infection is not heavy in the majority of cases. None had a heavy load of hookworms, which is not surprising as these people are not agriculturists. There was a slightly higher proportion with heavy loads of *Trichuris* and *Ascaris* in the patients with amebic dysentery than in the controls. In no instance in this investigation could dysenteric symptoms be ascribed directly to these helminths and our previous experience with *Trichuris* and *Ascaris* in dysenteric amebiasis has shown that removal of these worms has no effect on the course of the disease. We have been unable to obtain clear evidence indicating a relationship between the weight of infection with these helminths and the development of dysenteric amebiasis.

This study indicates that our patients with amebic dysentery have a higher incidence of *Trichuris* and *Ascaris* by comparison with the group with bacillary dysentery and with the controls. Either these parasites have some effect on the initiation of the disease, or the two conditions, that is to say, helminthiasis and amebic dysentery, have a similar epidemiology. Our present evidence cannot distinguish between these alternatives. Studies on a further 100 patients with amebic dysentery have shown a similar incidence and degree of helminth infection to that reported in detail in this paper. It is evident that more direct methods of investigation must be developed to determine precisely whether such helminths predispose to invasion by *E. histolytica*.

SUMMARY

Comparison of the incidence and degree of intestinal parasitism in Durban African males indicates that the incidence of protozoal infection in a control group is similar to that reported elsewhere but there is a significantly lower incidence in patients with bacillary dysentery and an even lower incidence in those with amebic dysentery. There is a high incidence of helminth infection in all three groups but those with amebic dysentery have the highest incidence of *Trichuris* and *Ascaris* infections. Heavy loads of *Trichuris*, *Ascaris* and hookworm are rare although the two former are slightly commoner in amebic dysentery.

It is concluded that, although no positive evidence exists, there is a possibility that the high incidence of *Trichuris* may predispose to invasion by *Entamoeba histolytica* although the association between the amebae and *Trichuris* and *Ascaris* may merely be due to common epidemiological factors of poor hygiene and lack of sanitation.

ACKNOWLEDGMENTS

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SCHISTOSOMIASIS MANSONI IN THE KENYA BABOON*

JACK P. STRONG,† HENRY C. McGILL, JR. AND JOSEPH H. MILLER

Departments of Pathology and Tropical Medicine and Medical Parasitology, Louisiana State University School of Medicine, New Orleans, Louisiana

Miller[1, 2] has reported the high prevalence of *Schistosoma mansoni* infections in baboons (*Papio doguera*) from Kenya, British East Africa. He also reported that G. S. Nelson had recovered adult worms and viable eggs identified as *S. mansoni* from the same species of baboon.[2]

The present report is based on a detailed study of the lesions of schistosomiasis in the animals from which Miller's data were derived. The distribution and characteristics of the lesions in baboons are described and compared with those of schistosomiasis in humans and in experimentally infected animals. The epidemiological significance of naturally occurring baboon infections with *S. mansoni* is also reviewed.

MATERIALS AND METHODS

One hundred fifty baboons of all ages and both sexes were sacrificed and necropsied shortly after being trapped from their native habitat in Kenya, British East Africa. The study was planned primarily to determine the prevalence of naturally occurring arterial lesions in these animals and to furnish base line data for future experimental work on atherosclerosis.[3] Parasitologic studies were included to supply information concerning the health of these animals.

The baboons were captured by a professional game trapper in the bush country of southeastern Kenya within a 10-mile radius of the confluence of the Athi and Kibwezi Rivers. They were anesthetized by intraperitoneal injections of sodium pentobarbital, and complete necropsies were performed immediately. Blocks of all major organs were fixed in 10% neutralized formalin. Histologic sections were prepared from paraffin-embedded blocks and stained with hematoxylin and eosin. At least one section of liver (and sometimes three or four) and a section of the colon were examined from each animal, as well as sections of heart, lung, spleen, pancreas, adrenal, kidney, stomach, small intestine, gonads, and thyroid. In many instances sections of salivary glands, skin, breast, muscle, and nerve were also examined. No serial sections were prepared from any blocks; but occasionally, when suspicious lesions were seen in the liver or colon, deeper cuts were made from the same paraffin block. This method undoubtedly leads to underestimation of the prevalence of infection since the samples examined represent only a small fraction of each organ.

The animals were divided into relative age groups on the basis of dental development as indicated in Table 1.

RESULTS

General observations. Schistosomiasis was not suspected on the basis of gross inspection of the organs of the baboons at the time of necropsy; however, there were many gross lesions due to other parasites. The lungs of practically all the animals except the youngest contained many gray and yellow nodules up to 5 mm in diameter and some minute scars, located mainly in the upper lobes of each lung. These lesions were due to lung mites, *Pneumonyssus sp.*

Most of the livers, especially those from adults, contained gray and yellow nodules from 1 to 4 mm in diameter, representing infections with *Hepatocystis kochi*. The intervening liver tissue was grossly normal.

The alimentary tract of most of the animals contained many parasitic nematodes and protozoa, and occasionally a few cestodes. The only gross lesions in the mucosa of the alimentary tract were focal areas of hyperemia in the colon and many small opacities grossly thought to be ulcers. The presence of colonic ulcers was not confirmed histologically in the majority of these cases. Where microscopic ulcers were present, no schistosome eggs, amebae, nor other organisms were found.

No evidence of splenic enlargement, esophageal

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† Senior Research Fellow (SF 150), U.S. Public Health Service.

TABLE 1

Prevalence of Schistosoma mansoni *infections in 150 baboons* (Papio doguera) *autopsied in Kenya, East Africa, July–August, 1958, as determined by histologic examination of tissue from complete autopsies*

State of dentition	Male						Female					
	No. of cases	Eggs in liver	Eggs in colon	Eggs other sites	Total cases infected	Per cent cases infected	No. of cases	Eggs in liver	Eggs in colon	Eggs other sites	Total cases infected	Per cent cases infected
No teeth erupted	0						3	0	0		0	0
Deciduous dentition	4	0	0		0	0	9	0	0		0	0
Developing permanent dentition	36	3	5	1*	5	14	29	5	5	1*	7	24
Completed permanent dentition, wear on 3rd molar												
Little or no wear	14	4	2		4	29	15	5	3		6	40
Slight	12	7	4		7	58	7	1	0		1	14
Moderate	6	3	3		3	50	4	2	2		2	50
Extreme	6	1	1		1	17	5	0	0		0	0
Total	78	18	15	1	20	26	72	13	10	1	16	22

* Small intestine.

varices, distended venous collaterals, or ascites was found. In short, there was no gross evidence of portal hypertension.

Histologic lesions of S. mansoni. The prevalence of histologic lesions containing schistosome eggs in the most commonly affected tissues is given in Table 1. Infection was limited almost exclusively to the liver and colon, with occasional involvement of the small intestine. The liver and colon were involved in almost an equal number of animals. In some animals eggs were detected in only one of these sites. In such cases the method of sampling was probably inadequate to demonstrate eggs in the apparently non-infected organ. *S. mansoni* eggs were present in the ileum in only two cases out of 20 in which sections of ileum were available. Sections of duodenum were examined in all cases but did not contain eggs. Schistosome lesions were not detected in lung, spleen, lymph nodes, kidney, adrenal, heart, thyroid, pituitary, urinary bladder, stomach, esophagus, salivary glands, pancreas, diaphragm, tongue, skin, breast, uterus, ovary, prostate, testis, or seminal vesicle.

Lesions of the colon due to *S. mansoni* were usually located in the mucosa or submucosa (Figure 1), but in one case were also present in the subserosa. Adult males and females of *S. mansoni* in the submucosal veins of the colon were detected histologically in only one animal (Figure 2). Eggs were present at all levels of the mucosa, both superficially and deep. They were usually in the interstitial tissue adjacent to venules and occasionally within venules. In some of the mucosal lesions there was very little or no inflammatory reaction about the eggs, but in most lesions there was an acute inflammatory reaction with neutrophilic and eosinophilic polymorphonuclear leukocytes and varying degrees of hyperemia. A small number of mucosal lesions showed pseudotubercle formation with epithelioid cells. In some of the eggs there was an intact miracidium. Varying degrees of degeneration of miracidia were seen, and occasionally only an empty egg shell remained. In the submucosa of the colon the tissue response to *S. mansoni* eggs was either an acute inflammatory reaction with neutrophils and eosinophils or pseudotubercle formation with epithelioid and giant cells.

In the ileum, *S. mansoni* eggs were detected in both venules and interstitial tissues of the villi. Frequently the eggs were surrounded by an acute inflammatory reaction (Figure 1F).

Various stages in the pathogenesis of the hepatic lesions of schistosomiasis were observed in sections of liver (Figures 3 and 4). Adult worms were found in the intrahepatic branches of the portal vein in two baboons. In several instances eggs were in the intrahepatic portal radicles. There was no cellular reaction to some of the eggs found within vascular spaces. About others

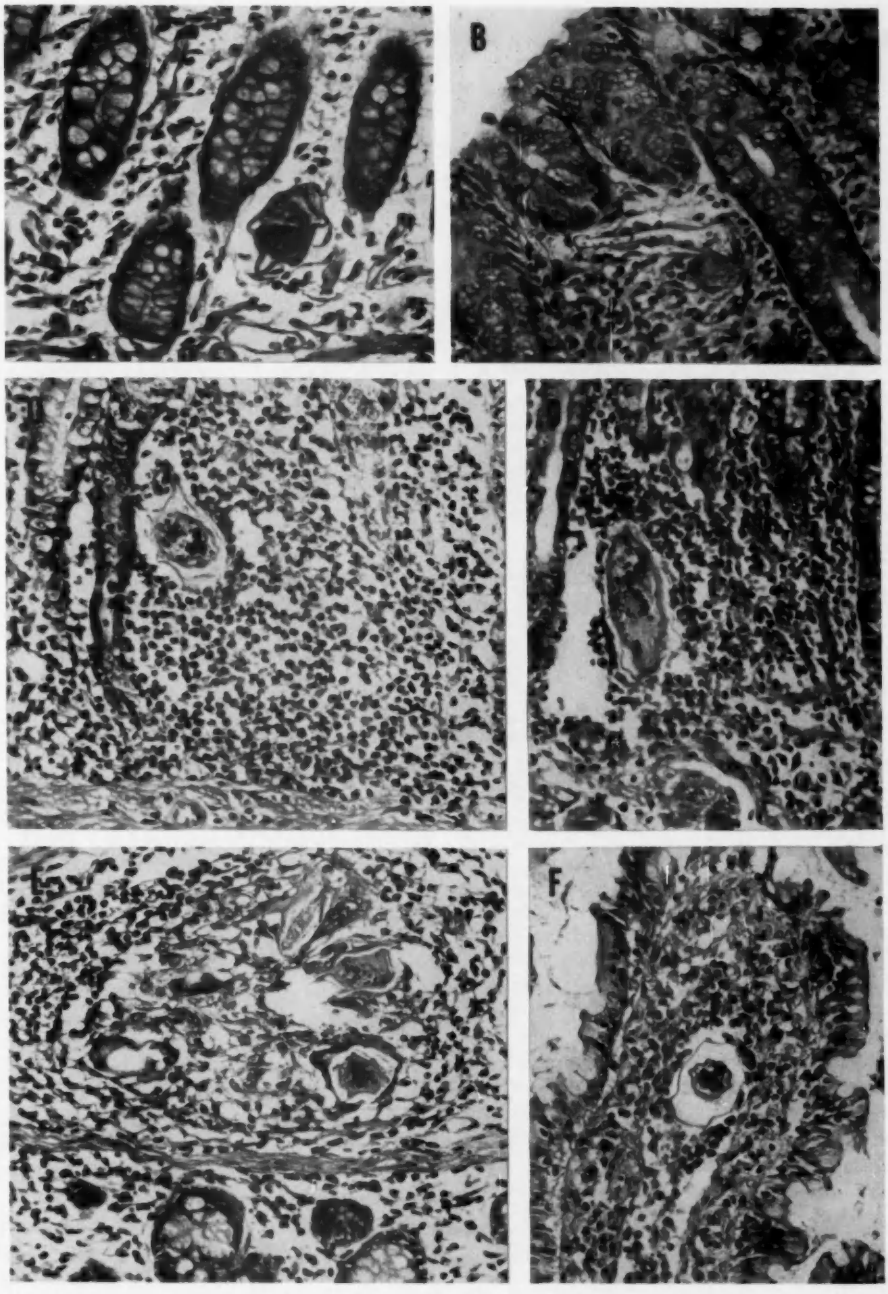

FIG. 1. *Schistosoma mansoni* lesions in the intestine (×240). A. Egg in venule deep in mucosa of colon without inflammatory reaction. B. Empty egg shell in upper mucosa of colon with mild giant cell reaction. C. Colonic lesion with egg surrounded by intense acute inflammatory reaction with neutrophilic and eosinophilic leukocytes. Note intact miracidium and visible spine. D. Egg containing intact miracidium deep within the mucosa of the colon. There is an inflammatory response with eosinophils and neutrophils. E. Pseudotubercle in submucosa of colon with eggs, giant cells, epithelioid cells, and lymphocytes. F. Ileum, egg in venule of villus. There are eosinophils and neutrophils about the egg within the venule.

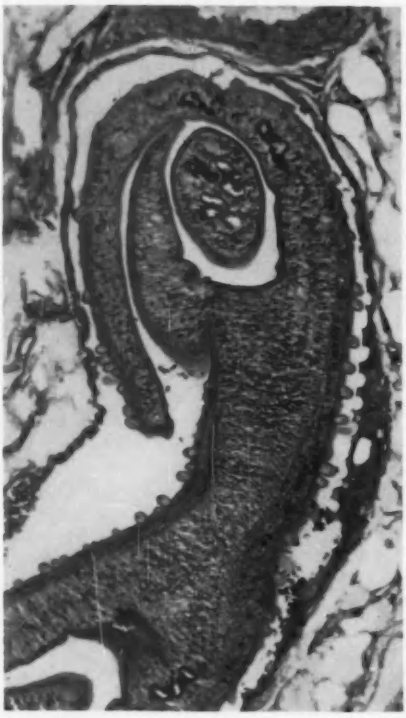

FIG. 2. *Schistosoma mansoni* male and female adult worms in submucosal vein of colon. Note tuberculated cuticle of male worm (×240).

there were numerous polymorphonuclear leukocytes. The majority of the hepatic lesions involved the portal zones. Reactions varied from acute inflammation with varying proportions of neutrophils and eosinophils to well formed pseudotubercles with giant cells, epithelioid cells, and an outer zone of lymphocytes. In most of the lesions the portal vein radicles were no longer visible. There was no appreciable fibrosis of the definite schistosome lesions. The general architecture of the liver lobules was preserved. Cirrhosis was not present.

DISCUSSION

The lesions of *S. mansoni* infection in the Kenya baboon are similar to those of mild human infection as classified by Koppisch[4] and Lichtenberg[5] with respect to the location of lesions, the type of inflammatory reaction, the absence of fibrosis or cirrhosis, and the presence of eggs in the feces in a small percentage of cases.[1] Baboons did not have the widespread distribution of eggs and lesions or the typical white clay pipe-stem cirrhosis of Symmers[6] as seen in severe human infections.[4, 5] This dissimilarity may have been due to various factors—the failure of severely infected animals to survive in nature, smaller or less frequent infecting doses of cercariae, or acquired immunity.

The schistosome lesions of the liver in baboons are similar to those found in mild human infections. Stages in the development of hepatic lesions observed in this study included the presence of eggs in the portal radicles with no reaction, impaction of eggs in the radicles with acute inflammatory reaction, intense acute inflammatory reaction about *S. mansoni* eggs in the region of the portal triad, and pseudotubercles. Many of the hepatic lesions demonstrated the process described by Lichtenberg[5] as "substitution by granuloma" in which the pseudotubercles completely replace the structures of the portal triad, particularly the portal radicles (Figure 4).

Light infection or the lack of some nutritional or other predisposing factor may account for the absence of cirrhosis in infected baboons. The former explanation seems to be the more likely.

The lower prevalence of infection in the oldest animals of this study has a parallel in human disease which has been pointed out by Newsome.[7] Presumptive evidence of human resistance to schistosomiasis is the decreased intensity and lower prevalence of infection in middle-aged and elderly individuals in hyperendemic areas.

Comparison of natural infection in baboons with experimental infection in other animals. This study demonstrates the susceptibility of the baboon in East Africa to natural *S. mansoni* infection. Newsome[7] reported the susceptibility of the baboon to experimental infection. He pointed out that the baboon infection was similar to that in the human, since baboons retain their infection for years, while in experimentally infected monkeys the infection dies out after a few months.

The similarity of the disease in baboons and humans also indicates the value of the baboon for experimental investigation of *S. mansoni* infection. Problems of species variation, which inevitably arise in the interpretation of experimental data, would be minimized by the use of a primate which reacts as the human does.

It has been demonstrated by other investigators[8-10] that there is a marked difference

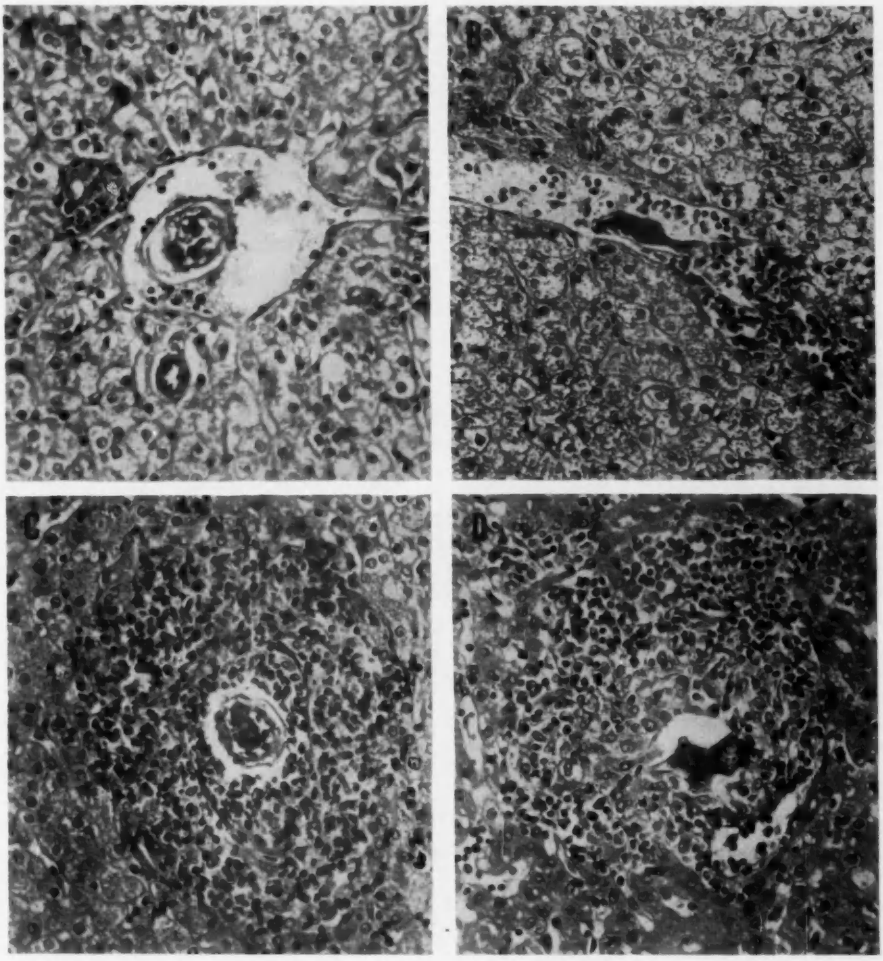

FIG. 3. Hepatic lesions of *Schistosoma mansqni* (×240). A. Egg in portal radicle with no cellular reaction. B. Egg with spine impacted in portal radicle, with an acute inflammatory reaction surrounding the egg. C. Egg in portal zone surrounded by eosinophils and neutrophils. D. Egg in portal zone surrounded by epithelioid cells, lymphocytes, and monocytes, forming a pseudotubercle. At the lower right of the lesion there is a venous channel which may represent a pre-existing portal radicle or a newly formed venule.

in species susceptibility to *S. mansoni*. In those hosts which are susceptible, the infection varies from a short abortive course to a fertile long term infection. Stirewalt *et al.*[8] reported that dogs, cats, rabbits, and guinea pigs are unsuitable for experimental infections. Mice, cotton rats, and hamsters were considered satisfactory for the laboratory maintenance of the parasite. Moore *et al.*[9] concluded that mice and hamsters are the most suitable common laboratory animals for experimental infections with *S. mansoni*; rats are unsatisfactory because very few worms develop to maturity. They considered guinea pigs and rabbits of value only for producing large numbers of worms. Eggs do not appear in the feces in these animals. Krakower *et al.*[11] stated that in the rat schistosomes mature and complete their life cycle in the intrahepatic branches of the portal

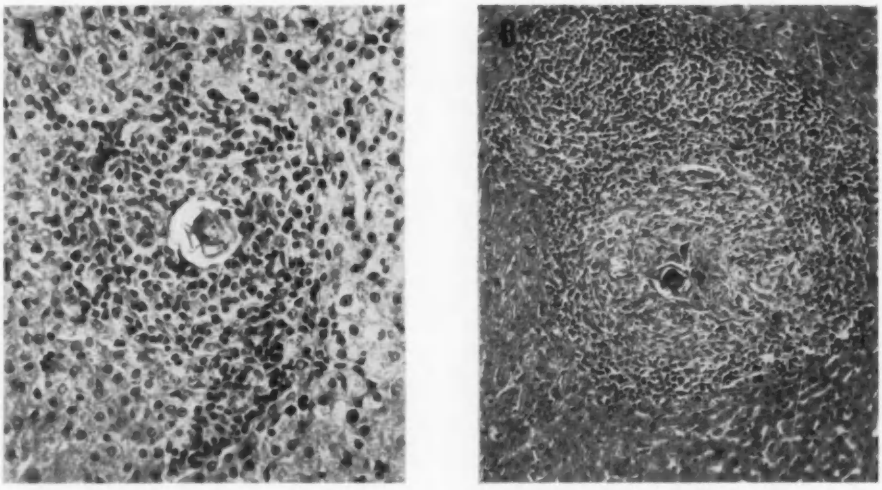

FIG. 4. Hepatic lesions of *Schistosoma mansoni*. A. Epithelioid cells, eosinophils, neutrophils, lymphocytes and macrophages about egg in portal zone in which the portal radicle cannot be identified and presumably has been obliterated (×240). B. Large pseudotubercle about egg in portal zone, showing complete replacement of the structures of the portal triad (×120).

vein and that only a few worms migrate to the mesenteric veins. Thus eggs are not passed in the feces. Meleney *et al.*[12] have pointed out that rabbits and albino rats are not favorable hosts for the normal development, reproduction, and long survival of *S. mansoni*. Kuntz and Malakatis[13] have studied the susceptibility of certain wild mammals of Egypt to infection by *S. mansoni*. The Nile rat, *Arvicanthis niloticus*, was the best experimental host of the animals evaluated in this study.

A number of workers have successfully infected monkeys with *S. mansoni*.[14-20] Primates which have been used include *Cercocebus sp.*,[17] *Cercopithecus aethiops*,[14] *Macacus mulatta*,[15, 16] *Macacus rhesus*[19, 20] and *Cebus sp.*[18] Some *Macacus mulatta* ceased passing eggs as early as 4 months after exposure.[15, 16]

It is apparent from reports of experimental infections that *S. mansoni* has a narrow host range. The most commonly used monkeys, *Macacus sp.*, are near the middle of the susceptibility spectrum for *S. mansoni*. However, the susceptibility of the baboon appears to be high. Thus, further experimentation with the baboon would be valuable in studying the mechanisms of resistance and immunity.

Epidemiological significance of naturally occurring S. mansoni *infections in the baboon.* Reservoir hosts for *S. mansoni* have been suggested in numerous publications. Natural infections in African rodents have been detected by Schwetz[21] and Teesdale and Nelson.[22] In South America the presence of naturally occurring *S. mansoni* infections has been reported in rats[23, 24] and rabbits.[25] Rodents are probably not significant reservoir hosts for *S. mansoni* in Africa since natural infections are rare and isolated.[21]

While primates have been widely used as experimental animals for the study of *S. mansoni* infections, there have been few systematic studies of African primates in their natural habitat to determine whether they are a reservoir of infection. A natural infection with *S. mansoni* in the West African green monkey (*Cercopithecus sebaeus*) on the Isle of St. Kitts in the West Indies was reported by Cameron in 1928.[26] McQuay[27] found a Guinea baboon, *Papio papio*, of unknown geographical origin, infected with *S. mansoni* in New Orleans. We have been unable to find any documented report of naturally occurring *S. mansoni* infection in subhuman primates in Africa. Natural infections with other species of schistosomes have been reported in primates.[28]

In their review on African schistosomiasis in 1953, Amberson and Schwarz[29] expressed the opinion that all African primates are susceptible to human schistosomiasis. They predicted, how-

ever, that only a few species would prove to be of importance as reservoirs. Prerequisites were considered to be a large population of animals in an area with water containing a suitable snail species. They suggested that two primates met these requirements: the green monkey, *Cercopithecus aethiops*, and the baboon, genus *Papio*. The same authors also predicted that the *Cebus capucinus apella* and *Cebus capucinus albifrons* of South America might serve as reservoir hosts for *S. mansoni* in that area. We might add to their prerequisites that inherent susceptibility to infection with *S. mansoni* also would determine whether an animal could serve as a reservoir host.

Statements by Amberson and Schwarz concerning the work of Blackie,[30] Archibald and Marshall[31] and le Roux[32] might be interpreted as indicating that naturally occurring infections with *S. mansoni* had been found in monkeys and baboons. Review of these publications indicates that such is not the case. Experimental infections of subhuman primates were produced by Blackie and by Archibald and Marshall, but none of these workers reported naturally occurring infections.

The baboons from southeastern Kenya which we studied are the first primates in which a significantly high prevalence of *S. mansoni* infections has been demonstrated. Since the baboon is numerous in practically all parts of East Africa, including areas of human habitation, it may be an important reservoir of infection for the parasite. Any measures directed at controlling schistosomiasis must take into account this possibility. Furthermore, the baboon and other primates in parts of Africa where human schistosomiasis occurs should be intensively investigated as possible reservoir hosts.

SUMMARY

One hundred fifty baboons from Kenya, British East Africa, had a high prevalence of infection with *Schistosoma mansoni*, indicating that the baboon may be an important reservoir host for this parasite.

The distribution of *S. mansoni* eggs in the tissues of the baboons is similar to that found in mild human infections and the pathogenesis of lesions in the colon and liver is also similar. Hepatic cirrhosis, however, was not observed in the baboon.

These studies, which point out many similarities between human and baboon infections, confirm Newsome's suggestion that the baboon is an excellent experimental animal for research in schistosomiasis. The baboon is superior in many ways to the common laboratory animals and also to other primates.

Measures to control human schistosomiasis should take into account the possibility that the baboon is an important reservoir host. Other primates should be examined in areas where human schistosomiasis exists to determine their role in host-parasite relationships.

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BLOOD AMMONIA LEVELS IN PATIENTS WITH ADVANCED HEPATIC FIBROSIS ASSOCIATED WITH SCHISTOSOMIASIS*

STANLEY W. HANDFORD†

Naval Medical Research Unit No. 3, Cairo, Egypt

Through the co-operation of the Egyptian Ministry of Health and the Director of the Abbassia Fever Hospital, Cairo, Egypt, an opportunity was afforded to make a limited number of measurements of some blood constituents in patients exhibiting the classical symptoms of terminal schistosomiasis often characterized by hepatosplenomegaly. In view of the resurgent interest in assessing the role of altered ammonia metabolism in the pathogenesis of advanced liver disease, it was of interest to compare blood levels of ammonia nitrogen (NH_3-N) and related compounds in these individuals with those of control subjects. Insofar as it was possible to determine, such measurements had not been reported previously on this clinical condition.

SUBJECTS

Twenty-nine patients with hepatosplenomegaly and with both *Schistosoma mansoni* and *S. haematobium* infections were studied. In addition to schistosomiasis these individuals suffered from malnutrition and were, or had been, infected with numerous other enteric parasites. All patients were ambulatory and under treatment. Of the number studied two were females. The age range was from 20 to 50 years with an average of 25. Fourteen of these patients were confined to the ward of the research unit to which they had been admitted for observation and/or treatment for brucellosis. (Fairly complete case histories are available on these subjects.) Fifteen cases were brought in from the adjacent fever hospital for sampling and testing. In only one case were there signs of encephalopathy; two involved esophageal varices and three, frank edema and ascites. Needle biopsy specimens of the liver from 15 of the patients were obtained and studied by the resident pathologist. The general appearance of a typical subject is shown in Figure 1; Figure 2 shows an extreme case.

Employed as controls were 14 sick Egyptians of the same range in age, social background, and nutritional status under treatment for brucellosis and multiple parasitism, including schistosomiasis but with no advanced associated disorders. In addition, 10 clinically healthy Egyptian and 10 American employees of the research unit, including one female, were studied.

METHODS

Blood for analysis was drawn into heparinized syringes from an antecubital or femoral vein early in the morning when the subjects were presumably in a postabsorptive state. In only two cases were additional samples available on successive days owing to local taboos concerning "blood letting." Following the collection of blood, BSP was injected as indicated.

Blood NH_3-N and urea nitrogen were determined immediately by the microdiffusion methods of Seligson;[1, 2] blood glutamine by the method of Boulanger and Osteux;[3] pyruvic and alpha keto-glutaric acids by conversion of their 2-,4-dinitrophenylhydrazines separated by paper chromatography and measured colorimetrically by the method of El Hawary and Thompson;[4] and plasma proteins by the Biuret method. BSP retention, thymol turbidity and bilirubin levels were determined by standard methods.[5]

RESULTS

A comparison of the results of liver function tests (Table 1) between patients with liver disease and control patients indicates that in this respect a valid dichotomy exists between the two groups. (Liver biopsies were not available on the "sick Egyptian" controls.) The slight elevation in the control group is attributable to two borderline cases included; since the test group also included two borderline individuals insofar as the function tests were concerned, it was decided not to omit these individuals

* The opinions or assertions contained herein are the private ones of the author and are not to be construed as official or reflecting the view of the Navy Department or the naval service at large.

† Present address: Naval Medical Research Institute, Bethesda 14, Maryland.

FIG. 1. Typical case of advanced schistosomiasis with hepatomegaly.

FIG. 2. An extreme case of schistosomiasis with ascites, esophageal varices and general debility.

from the study. Function tests were not done on the two "healthy" control groups, since none presented a history of liver disease.

From the data summarized in Table 2, however, it is apparent that there is no significant difference in the other parameters measured between the test and control subjects. The observed reversal in the albumin-globulin ratio is indubitably a reflection of chronic disease and malnutrition. In every case the globulin fraction is greater than the albumin. This is in agreement with the observation of Ragheb.[6]

A consideration of individual cases is little more revealing. For example in No. 805 the average value for blood NH_3-N based upon three determinations made on successive days was 0.268 mg%, a figure 168% above the accepted normal value; urea nitrogen and plasma glutamine were within normal limits; but pyruvic and alpha keto-glutaric acid levels were elevated above normal. This individual exhibited a BSP retention of 36%, thymol turbidity of 8 units, bilirubin of 3 and 4.6 at 1 and 10 minutes, respectively, and an A/G ratio of 0.29. Of the 29 subjects studied this is the only one who appeared to present any sign of neurologic disorder. (In this case what appeared to be confusion and disorientation as to time and place could easily have been derived from a generally low mental status. No more than the most superficial neuropsychological evaluation of the patients was practicable under the circumstances of the study.) In contrast is No. 796 who presented a BSP retention of 50.6, thymol turbidity of 12.3, bilirubin 7.8 and 12.8, marked edema with ascites and bleeding esophageal varices but, aside from the usual elevation in plasma globulin, showed no deviation from normal in the other biochemical variables measured. Similarly No. 202, who died from bleeding

TABLE 1

Average results of liver function tests on patients with hepatic fibrosis and advanced schistosomiasis (I) and control patients with brucellosis and multiple parasitism (II)

	BSP % retention	Thymol turbidity units	Bilirubin (direct and indirect van den Bergh)	
			1 minute	10 minutes
Normal	(0–7)	(0–5)	(0.1)	(0.3)
I. N = 29	15.2	8.6	0.78	1.98
Range	(2–20)	(3.2–33)	(0.08–7.8)	(0.2–12.8)
II. N = 14	4.5	5.9	0.31	0.84
Range	(3–9)	(4–10)	(0.12–0.72)	(0.28–1.68)

TABLE 2

Summary of tests made and results obtained on residents of Cairo, Egypt

		Blood NH_3-N mg%	BUN mg%	Plasma glutamine mg%	Pyruvic acid mg%	Alpha keto-glutaric acid mg%	Plasma proteins (g %)		
							Alb.	Glob.	A/G
Normal range		0–0.1	9–19	5–12	0.44–0.64	0.16–0.19	4.3–5	1.1–3.1	1.50
Healthy Americans (NAMRU-3 personnel)	No.	10	10	5	9	4	10	10	
	Ave.	0.055	10.6	5.5	0.23	0.20	3.84	3.48	1.11
	Range	0.04–0.08	7–13	3–7	0.15–0.31	0.1–0.35			
Healthy Egyptians (NAMRU-3 personnel)	No.	10	10	10	10	10	10	10	
	Ave.	0.064	10.0	13.6	0.28	0.23	3.69	3.78	0.98
	Range	0.04–0.08	6–14	9–16	0.08–0.57	0.1–0.37			
Egyptians with brucellosis, multiple parasitism	No.	14	11	9	14	14			
	Ave.	0.069	11.6	7.2	0.38	0.16			
	Range	0.04–0.1	5.4–20	3–11	0.20–0.70	0.12–0.24			
Egyptians with schistosomiasis, hepatic fibrosis*	No.	29	30	25	22	22	29	29	
	Ave.	0.065	12.5	9.6	0.43	0.25	2.48	5.58	0.46
	Range	0.015–0.290	7–37	6–14	0.12–0.80	0.12–0.40			

* Ascites, edema in non-comatose patients with no apparent encephalopathy.

from esophageal varices and general debility, had shown a blood NH_3-N of .028 mg% 2 days previously. At autopsy a liver of 1280 g showed, on all surfaces, coarse hobnail-type cirrhosis with large, yellow nodular areas of regenerative hyperplasia. Microscopic examination revealed (1) pulmonary schistosomiasis due to *S. haematobium* invasion; (2) coarse periportal cirrhosis of the liver associated with the same organism; (3) advanced congestive fibrosis of the spleen; (4) acute ulceration and congestion of the stomach with esophageal varices; (5) colon and vermiform appendix inflamed with chronic granulomatous ulceration and polyposis infested with ova of *S. haematobium;* (6) kidney hydronephrotic and (7) ureter, bladder and prostate inflamed, containing numerous ova of *S. haematobium* in the fibrotic tissues.

Needle biopsy specimens of liver obtained from 15 of the test subjects all showed, with some variation in degree, periportal fibrosis associated with schistosomiasis.

DISCUSSION

It is beyond the scope of this study to discuss the causal relationship between schistosomal invasion of the hepatic portal system and the high incidence of hepatic cirrhosis among Egyptian fellaheen. This aspect of the problem has been amply and convincingly covered by the comprehensive study of Ragheb.[6] His work leaves little doubt that malnutrition and schistosomiasis of both types endemic in Egypt (*S. mansoni* and *S. haematobium*) may be singly or jointly responsible for the condition. Further, despite previous observations to the contrary (see Ragheb[6]), it now seems clear that parenchymal damage,[7] as well as fibrotic changes, often characterizes this type of cirrhosis. The measurements made in the present study are simply a small addendum to the observations of Ragheb.[6]

In view of the inconsistent and inconclusive results reported in the literature on the causal relationship between elevated blood NH_3-N levels and hepatic insufficiency, it is not surprising in the present instance that no correlation is shown. From the clinical standpoint the present status of the problem has been summarized by Sherlock[8] who observes: "Blood ammonium levels are frequently elevated in hepatic coma but 10 per cent of values are in the normal range regardless of the degree of neurological involvement. . . At the present time it is impossible to decide whether the elevated blood ammonium levels reflect mainly the amount of portal venous

blood reaching the systemic circulation without being adequately metabolized by the liver or whether the ammonium is an important cerebral intoxicant in patients with liver disease."

Recent in vivo experimental studies[9, 10] have shown that in addition to the synthesis of urea by the liver, the rapid synthesis of glutamine from ammonia and glutamic acid by brain and other tissues is an important pathway for the rapid clearance of ammonia. It appears that urea synthesis takes care of a fixed amount of ammonia and continues to do so in face of severe liver damage, whereas the major mechanism for ammonia clearance is glutamine synthesis. This reaction occurs very rapidly in the tissues of the organism. However, since it is generally accepted that blood and plasma levels of glutamine and related substances do not necessarily reflect the concentrations of these substances in other tissues, it may be that the measurements made on blood in the present study are inadequate to reflect changes in the organism as a whole. It is conceivable that in cases of hepatic failure exhibiting no encephalopathy nor marked elevation in blood NH_3-H level that the synthesis of glutamine by the brain continues at an efficient rate with replacement of glutamic and alpha keto-glutaric acids at a rate adequate to prevent any interference with aerobic glycolysis necessary to its metabolism. Conversely, when the substrate is not renewed at a rate commensurate with the metabolic requirements of the brain, the signs and symptoms of hepatic coma and other encephalopathies may supervene.

SUMMARY

Blood NH_3-N, urea, glutamine and keto acids were measured in 29 patients with hepatic fibrosis associated with advanced schistosomiasis and malnutrition. Routine hepatic function tests (BSP clearance, thymol turbidity and bilirubin index) were elevated in all cases. Despite evidence of liver failure and pathologic involvement, none of the biochemical parameters measured were significantly elevated relative to the controls.

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A CASE OF DIROFILARIASIS INVOLVING THE LUNG

GRAYSON F. DASHIELL

Metropolitan Hospital Clinics, Detroit, Michigan

It is the purpose of this paper to report a case of human infection with a worm of the genus *Dirofilaria* involving lung tissue. This anatomical site of infection has not been noted in previous reports. While reporting two new cases in 1957, Faust reviewed the literature and analyzed 37 known cases.[1] Since then Sams and Beck[2] have reported two others, Lenth[3] has reported one, and Jung and Harris have reported one.[4] The conjunctivae and subcutaneous tissues are most commonly involved. In a few instances the worms have been found in the heart or adjacent vessels, the abdominal cavity and mammary gland.

CASE REPORT

Mrs. F. H., a 57-year-old white woman, was found to have a nodular density about 1 cm in diameter in the right upper lobe of the lung by x-ray in December, 1959. A previous film taken in October, 1958, was normal. Further x-ray studies demonstrated the density to be in the anterior segment of the lobe. Because of the high incidence of malignancy in lesions with this location the involved area was resected. The specimen, surprisingly, was found to contain "worms." Ten sections for microscopic study, sent to Dr. Paul Beaver of Tulane University, were identified as parts of a single worm belonging to the genus *Dirofilaria*. Unfortunately, other parts of the worm or sections through other areas could not be obtained. In the 14 months prior to admission the patient had developed hypertension and mild cardiac decompensation. Ventricular extrasystoles, not noted previously, were consistently found. However, she responded well to treatment and later pursued normal activities. Cardiac enlargement was never found by x-ray. Between October, 1958, and December, 1959, electrocardiograms showed premature ventricular beats and nonspecific ST segment changes. Although the patient had resided in Detroit for 20 years, it was learned that she had made 12 trips to South Carolina in the last 8 years. One of these was made in the interval between October, 1958, and December, 1959, and that trip was in May, 1959. The patient did not recall any febrile illness during or after her South Carolina visits nor did she recall any skin or ocular lesions.

The worm. Transverse and longitudinal sections of a worm in a necrotic pulmonary artery were present in six sections on four slides. Although the worm was dead and had lost the normal architecture of internal structure, it was possible to recognize remnants of the muscles, lateral chords and reproductive system (Figure 1). Transverse sections ranged in diameter from 170 μ to 290 μ with an average diameter of about 200 μ. The cuticle, 4 μ to 6 μ in thickness and expanded laterally to project slightly into the lateral chords, appeared to have at least three distinct layers (Figure 2): an outer, relatively thin and smooth layer with no evidence of longitudinal ridging; a thicker fibrous middle layer composed of variously oriented fibrous elements; and a thinner lamellar layer at the base of the cuticle. In some sections there appeared to be a fourth layer between the fibrous and lamellar layers, most evident in the thickened lateral areas adjacent to the chords. The lateral chords were poorly preserved but distinguishable from the muscles. They appeared to be very broad and expanded into the pseudocele. Nuclei were not evident. The muscles of the body wall, extending inward to the central organs, consisted of relatively few cells having distinct alveolar and fibrous portions. In all sections of the worm one, two or three tubular structures of the reproductive system, apparently female, were evident. The gut was not recognizable.

Laboratory data. A 10-ml sample of blood taken 10 weeks after operation was fixed in 2% formalin, concentrated, and the entire sediment examined. No microfilariae were found. Thin and thick films (stained with hematoxylin) collected at various hours in the day and night on five occasions over a 4-day period were all negative for microfilariae. Filarial complement fixation and indirect hemagglutination tests for *Ascaris* and *Toxocara* were negative. Repeated stool examinations revealed no parasites. Findings on urine

FIG. 1. Transverse section of worm showing general features and location in necrotic pulmonary artery. × 200.

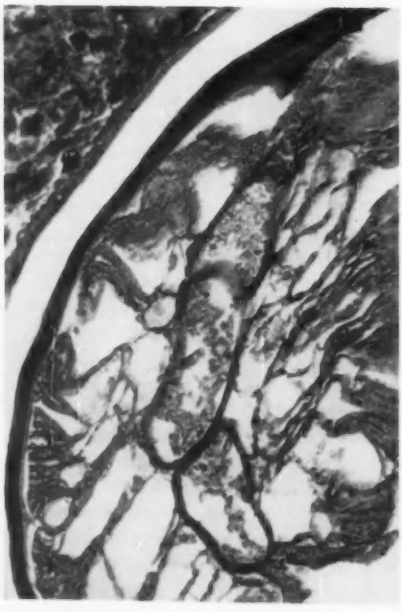

FIG. 2. Portion of worm demonstrating cuticle, muscles, and uterus. × 565.

and blood examinations prior to operation and later were within normal limits.

COMMENT

The thick, laminated cuticle, broad lateral chords, large muscle cells and apparent dimensions indicate that the worm in this case was a *Dirofilaria*. Absence of ridges on the cuticle, the location of the worm in a pulmonary artery, and its size suggest the possibility of its being *Dirofilaria immitis*, the common heartworm of dogs. It has been suspected in other cases[1] that *Dirofilaria* removed from human tissues and morphologically resembling *D. immitis* were indeed that species, although they were in most cases identified as *D. conjunctivae*.

The association of a dead filarial worm in the lung with the cardiac symptoms and the findings in this case raises the question of causal relationship of this worm and others that may have been unobserved in other locations. Although the presence of living worms is not ruled out by the negative filarial complement fixation test, this finding, along with other negative findings over a period of 7 months, indicates that only one worm was present. Although possibly not, this worm probably was acquired in South Carolina.

SUMMARY

Chest x-ray detected a lesion in the lung of a Detroit resident with cardiac symptoms. In the resected cyst-like lesion a filarial worm was found dead in a necrotic pulmonary artery. It was identified as *Dirofilaria*, apparently a female, and was thought to have been acquired in South Carolina.

ACKNOWLEDGMENT

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ACTION OF AQUEOUS SOLUTIONS OF IODINE ON FRESH VEGETABLES AND ON THE INFECTIVE STAGES OF SOME COMMON INTESTINAL NEMATODES*

PRAYUTH THITASUT†

Department of Tropical Medicine and Public Health, Tulane University School of Medicine, New Orleans, Louisiana

In some regions of the world where night soil is used as fertilizer for crops, vegetables may be contaminated simultaneously with infective stages of *Ascaris*, hookworms, and other helminths. In these regions vegetables are mainly consumed raw, partially cooked or pickled. It has been found that larvae of hookworms and eggs of other helminths can be demonstrated on marketed vegetables.[1] *Ascaris* eggs firmly adhere to certain kinds of leafy vegetables,[2] and may remain viable even after pickling in brine or in other substances.[3-5]

It has been shown that in certain concentrations a number of chemicals kill the infective stages of *Ascaris* and other worms and could have some usefulness in the control of intestinal helminths,[5-8] but no substance has been found suitable for use as a disinfectant of fresh vegetables.

A number of studies have demonstrated the killing action of aqueous iodine on bacteria,[9, 10] fungi,[11] and protozoa.[12] Solutions of free iodine have been used for disinfecting food utensils[13] and small or large quantities of water.[14-16] Iodine in aqueous solutions is more palatable and less irritating to the mucous membranes than is chlorine.[15] A study of humans consuming iodized water (12 to 19 mg per day) for a period of 6 months revealed no untoward effects.[17] These observations suggested the possible merit of aqueous iodine as a disinfectant of fresh fruits and vegetables.

The present study was planned to determine the amount of iodine in water necessary to kill the infective stages of *Ancylostoma caninum*, *Necator americanus*, *Strongyloides stercoralis*, *Ascaris lumbricoides*, *Toxocara canis*, and *Trichuris trichiura*, at different contact times and temperatures, and also to determine the effects of aqueous iodine on some of the more common fresh vegetables. Experiments on the infective eggs of *T. trichiura* proved to be impractical, as no reliable test of larva viability could be devised. *Trichuris muris* was therefore substituted for *T. trichiura*.

MATERIALS AND METHODS

Infective *Ancylostoma* and *Necator* larvae were obtained from charcoal cultures by the damp-pad technique,[18, 19] and from test-tube cultures in the manner described by Harada and Mori.[20] Larvae obtained by either method generally retained the protective cuticular sheath. Larvae of *S. stercoralis* were cultured in a thin layer of feces on filter paper in a Petri dish containing enough water to maintain dampness. Infective stages of *Ascaris*, *Toxocara* and *Trichuris* were obtained by incubation of washed and concentrated eggs in 120-ml screw-cap specimen jars containing distilled water at a depth of 0.5 cm; the water was changed daily to prevent overgrowth of bacteria and fungi.

Solutions of iodine in various concentrations were prepared by dissolving commercially prepared tablets‡ (Globaline® or Heliogen®) in distilled water. The approximate concentrations of free iodine before and after the tests were determined by titration. One drop of a concentrated suspension of the infective stages was added to a

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† Present address: Department of Pathology, Siriraj Hospital Medical School, Bangkok, Thailand.

‡ Globaline® tablets, produced by Abbott Laboratories (North Chicago, Illinois) for disinfection of small quantities of water, each gave approximately 8 ppm titrable iodine in 1 liter of water; tablets of Heliogen® (Heliogen Products, Inc.), supplied by S. B. Penick & Company (50 Church Street, New York City) for use in large volumes of water, each gave about 250 ppm titrable iodine in 1 liter of water. An iodine test kit (starch-iodide), manufactured by Iode Chemical Company (Dublin, Ohio), was used for the titrations.

60-ml glass-stoppered bottle completely filled with the test solution. For *Ancylostoma*, *Necator* and *Strongyloides* observations on motility were made every 5 or 10 minutes under hand-lens magnification (5 ×); when apparently immobilized, the larvae were observed under higher magnification; iodine staining of the larvae was used as evidence of death. The eggs of *Ascaris*, *Toxocara* and *Trichuris* were observed by direct microscopic examination at 5- to 20-minute intervals to determine roughly when larvae within the eggs became non-motile and stained with iodine. As a confirmatory test of viability and infectivity, samples of treated (and promptly washed) and untreated eggs were inoculated into mice. If infective, the ascarid larvae were readily found at 4 to 7 days in the liver and lungs, and *Trichuris* larvae at 6 to 8 days in the cecum of the mice. The experiments were carried out with various concentrations of iodine at 25°C to 30°C (room temperature), at 15°C and at 20°C maintained as closely as possible by ice-pack and water-bath, and at 37°C in an incubator. Iodine concentrations were determined at the beginning and at the end of each experiment. Controls in distilled water were run with each experiment. At critical concentrations the tests were repeated at least three times with eggs or larvae from different cultures.

RESULTS

Preliminary tests indicated that at least 30 ppm of titrable iodine would be required to kill any of the test organisms within 1 hour at room temperature. It was also noted in preliminary trials, and in all of the later tests, that within 1 hour, the longest period of exposure, there was no significant reduction in the concentration of available iodine in the test solutions. Solutions containing the same amounts of titrable iodine gave essentially the same results whether prepared from one or the other of the two commercial products tested.

Larvae. In solutions containing 60 or more ppm titrable iodine all of the hookworm and *Strongyloides* larvae were killed within 10 minutes at room temperature (Table 1). Survival periods were shorter at 15°C, longer at 37°C. Of the three kinds of larvae tested, *Ancylostoma* was most resistant and *Strongyloides* least resistant to the iodine solutions at all concentrations and at all temperatures tested. At 15°C to 30°C *Strongyloides*, *Necator*, and *Ancylostoma* larvae were killed almost immediately in iodine solutions containing 50, 60, and 70 ppm respectively. Even the most resistant larvae (*Ancylostoma*), at the most favorable temperature (37°C), survived less than 15 minutes in solutions containing 70 ppm of free iodine. Infective larvae of *Strongyloides spp.* from rhesus monkeys and from nutria (*Myopotamus coypus*) were slightly more resistant than those of *S. stercoralis* but less resistant than those of *Necator*.

TABLE 1

Time required to kill infective larvae of Necator americanus, Ancylostoma caninum, *and* Strongyloides stercoralis *in various concentrations of aqueous iodine solution at various temperatures*

Iodine ppm	Minutes required to kill larvae at			
	15°C	20°C	25–30°C	37°C
	Necator			
35	30–40	30–40	50–60	*
40	20–30	20–30	40–50	*
45	20–30	20–30	30–40	50–60
50	5–10	5–10	10–15	25–30
60	1–5	1–5	1–5	15–20
	Ancylostoma			
40	30–40	30–40	*	*
45	20–30	20–30	30–40	*
50	10–15	10–15	20–30	45–50
60	5–10	5–10	5–10	20–30
70	1–5	1–5	1–5	10–15
	Strongyloides			
35	20–30	20–30	40–50	50–60
40	10–15	10–15	15–20	30–40
45	5–10	5–10	5–10	10–15
50	1–5	1–5	1–5	5–10

* Some or all larvae survived more than 1 hour.

No special tests of viability were required for the hookworm and *Strongyloides* larvae. As soon as they became immobilized the larvae absorbed the iodine and became distinctly yellow or brown in color.

Eggs. Infective larvae in eggs of *Ascaris*, *Toxocara*, and *Trichuris* were much more resistant to the iodine solutions than were the free larvae of hookworms and *Strongyloides* (Table 2). A concentration of 250 ppm was required to kill the *Trichuris*, the most resistant, in less than 15 min-

TABLE 2

Time required to kill infective larvae (in eggs) of Ascaris lumbricoides, Toxocara canis, *and* Trichuris muris *in various concentrations of aqueous iodine solution at various temperatures*

Iodine ppm	Minutes required to kill larvae at			
	15°C	20°C	25–30°C	37°C
Ascaris				
50	30–45	30–45	*	*
60	20–30	20–30	50–60	*
70	15–20	15–20	30–40	45–60
80	10–15	10–15	20–30	40–50
90	5–10	5–10	10–15	20–30
100	1–5	1–5	5–10	10–15
Toxocara				
80	30–40	30–40	50–60	*
90	20–30	20–30	40–50	50–60
100	10–15	10–15	15–20	20–30
120	1–5	1–5	5–10	10–15
Trichuris				
180	60	60	60	*
200	30–40	30–40	50–60	50–60
220	20–30	20–30	20–30	50–60
240	10–15	10–15	15–20	30–40
250	10–15	10–15	10–15	20–30

* Some or all larvae survived more than 1 hour.

utes at room temperature. The *Ascaris* and *Toxocara* were killed in 10 minutes or less by solutions containing less than half as much iodine, 120 and 100 ppm respectively, at room temperature. At the different concentrations, survival was increasingly longer at the higher temperatures within the tested range of 15°C to 37°C (Table 2).

Fresh vegetables. Several kinds of common leafy and rooty vegetables such as cabbage, collard greens, lettuce, spinach, celery, parsley, radishes, carrots, turnips and potatoes were exposed to iodine concentrations of 60 ppm and higher for 20 to 60 minutes at room temperature. There was no detectable permanent change in the flavor or general appearance of these vegetables. A change in color to blue occurred in potatoes with the skins broken or removed. After 15 minutes of treatment with solutions of 500 ppm the lettuce and parsley were slightly wilted but no change in their flavor could be detected. In concentrations up to 1,000 ppm the iodine did not penetrate the deeper layers of the unbroken skin of rooty vegetables and there were no detectable permanent changes in flavor or appearance. Titrations to determine the reduction of free iodine in solutions used to test the direct action on vegetables were not made but no color change was detectable.

DISCUSSION

Chandler[21] found that free iodine in a solution of potassium iodide instantly killed unsheathed hookworm (*Uncinaria polaris*) larvae. He also stated that various ascarid and strongylid eggs and larvae were immediately killed in Lugol's solution having a free iodine content of about 500 ppm.

In the experiments herein reported, the infective larvae of hookworms and *Strongyloides* were killed in 5 to 10 minutes when they were exposed to aqueous iodine solutions of 50 to 60 ppm at any temperature between 15°C and 30°C. The concentration of iodine necessary to kill the larvae within a given time was appreciably decreased at lower temperatures and increased at higher temperatures. The difference in results from exposures at low and high temperatures cannot be explained at present. It apparently did not result from a difference in the concentration of available free iodine. This order of effectiveness with respect to temperatures is the reverse of that found for other toxic chemicals,[22] and for iodine when applied to lower organisms.[23, 24] The data obtained on the lethal action of various concentrations of aqueous iodine were similar for free larvae and for larvae within the egg shell; but for killing the latter approximately twice as much iodine was required. The egg shell apparently provides considerable protection for the larvae. To promptly kill the larvae in the most resistant eggs a concentration of 250 ppm was necessary. From the practical standpoint, however, the requirement of 250 ppm does not seem to be objectionable since twice that amount did not damage fresh vegetables.

Solutions of sufficient strength to destroy the infective stages of the common intestinal nematodes in 10 minutes or less at temperatures within the normal range of tropical climates are distinctly brownish yellow and have a relatively weak but detectable iodine odor. Weaker solutions are adequate for destroying the common enteric bacteria and protozoa.[12, 24] Even for solu-

tions containing more than enough iodine to kill the most resistant of the tested organisms (*Trichuris*), the cost is nominal.

All tests in the present study were conducted on washed eggs and larvae completely exposed to the disinfectant. On contaminated fruits and vegetables, eggs, larvae and other infectious organisms possibly would be protected from the action of iodine by waxes and other substances. Thus, although the use of aqueous iodine as a disinfectant for fresh vegetable foods appears to be feasible, its applicability to practical situations cannot be taken for granted. At least, precautions should be taken to remove protective particles and masses from the vegetables and to expose all surfaces to the disinfectant.

SUMMARY

Investigations were made on the concentrations of aqueous solutions of iodine required to kill the infective stages of *Necator americanus*, *Ancylostoma caninum*, *Strongyloides stercoralis*, *Ascaris lumbricoides*, *Toxocara canis* and *Trichuris muris*. Solutions containing 70 ppm free iodine, prepared by dissolving tablets of Globaline® or Heliogen® in distilled water, killed the infective free larvae of hookworms and *Strongyloides* within 5 minutes or less at temperatures ranging from 15°C to 30°C. To kill infective larvae in the eggs of *Ascaris*, *Toxocara* and *Trichuris* under similar conditions concentrations of 100, 120, and 250 ppm, respectively, were required. Killing action was more rapid at the low than at the high temperatures. Solutions at concentrations up to 500 ppm did not adversely affect the flavor or appearance of any of the common types of leafy or rooty vegetables. Solutions of sufficient strength to kill the eggs and larvae of these worms, and at the same time to destroy the common enteric microorganisms, are easily prepared and therefore appear to be suitable for the disinfection of vegetables to be consumed in the fresh state.

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STUDIES ON RATES OF RECOVERY OF *SHIGELLA* FROM DOMESTIC FLIES AND FROM HUMANS IN SOUTHWESTERN UNITED STATES

C. S. RICHARDS, W. B. JACKSON, T. M. DeCAPITO AND P. P. MAIER

Communicable Disease Center, Public Health Service, U. S. Department of Health, Education, and Welfare, Phoenix, Arizona

The studies reported here were undertaken to determine whether or not suitable indices of the prevalence of *Shigella* in a community could be obtained from the rate of recovery of these organisms from domestic flies. This investigation was part of a program designed to develop methods for the control of diarrheal diseases in the Southwest. Generally the rectal swab technique described by Hardy and Watt[1] is preferred for determining rates of *Shigella* infection in community surveys. This method is efficient but expensive, and it also has the disadvantage of requiring professional field personnel and, in some instances, interpreters. In addition, this technique is sometimes limited by parental or community resistance and inability to find individuals at their homes. A sampling method that does not require professional field personnel and does not involve collection of specimens from persons has advantages in many studies.

The possibility of obtaining an index of *Shigella* prevalence by recovery of organisms from flies was considered because of the predilection of these insects for human excrement and because of their role in transmission of infections. The dry climate and the scarcity of other fly attractants in the study area were conducive to more frequent association of flies and human feces than in other environments where a variety of attractants was present. Previous studies, reviewed by Lindsay and Scudder,[2] implicated flies in the transmission of diarrheal disease. The first controlled study to demonstrate the effect of nonbiting flies on the incidence of diarrheal disease, particularly shigellosis, was conducted by Watt and Lindsay.[3] Although no successful attempts to isolate *Shigella* from flies in nature have been reported, laboratory studies by Hawley *et al.*[4] have shown that viable *Shigella* organisms could be recovered from flies for several days after ingestion of these organisms.

METHODS

The study was conducted from May, 1954, through December, 1955, in two Indian villages, Sacaton and Guadalupe, in an arid region of southcentral Arizona where measurements of the incidence of diarrheal disease and prevalence of *Shigella* were in progress. Flies were collected weekly from designated areas in the villages. In general, each collection represented a separate site. Representative areas were selected for examination each week in a manner that enabled complete coverage of the towns every month. Outdoor collections were made with a cone net,[5] and indoor collections were made with a portable vacuum cleaner.[6] Flies were transferred from the collecting devices to wire holding cages, immobilized with CO_2 gas, and then identified, counted, and stored in transparent polystyrene vials. Large collections were subdivided into pools of 20 flies or less per vial. Capped vials then were buried in powdered CO_2 ice in a portable refrigerator insulated with 8 inches of styrofoam. Records were maintained of the number of flies per pool and collection, genus, date, time, location, and attractant from which collections were made. After each collection, the holding cage was immersed in ethyl alcohol (95%) for 10 minutes and dried to prevent carry-over of *Shigella* organisms; the cone net was treated similarly between collection areas.

Flies were kept frozen until they could be processed in the laboratory, usually within 24 hours. Each pool of flies was thawed quickly and ground in 2 ml of warm *Shigella-Salmonella* (Difco) broth with a sterile mortar and pestle. (Nutrient broth was used the first 10 months of this study.) The resulting mixture was centrifuged at 15,000 rpm for 20 minutes and the supernatant discarded. The residue was removed to an SS agar plate with a wire loop and spread with a straight wire on this and two additional plates. The plates were examined after incubation for 24 hours at 35°C and re-examined after additional incubation for 24 hours at room temperature. Colonies resembling *Shigella* were picked and transferred to triple sugar iron (TSI) agar tubes. Additional biochemical reactions in various media

and serological tests were used to type *Shigella* according to standard methods.

Data on attractants, genus of flies, and density of fly populations were augmented by grill counts[7] in nine selected blocks in the two towns.

Concurrently with fly collecting, rectal swab samples were collected from preschool children, 1 to 5 years of age, and processed for isolations of *Shigella* according to methods devised by Hardy and Watt.[1] During part of the study, samples of privy contents were obtained with a swab, plated in the field, and cultured for *Shigella* in the laboratory.

RESULTS

Recovery of Shigella *from flies.* Collections of flies were made outdoors in Sacaton and Guadalupe for 20 months from May, 1954, through December, 1955. Table 1 shows by months the average grill count of flies, number of collections, number of flies collected, number of pools examined, and number of *Shigella* isolations. From Sacaton, 2,455 pools containing 14,054 flies yielded 16 isolations of *Shigella*. From 3,970 pools containing 51,219 flies collected in Guadalupe, 53 isolations were made. The rate of recovery in both towns was about 0.10 *Shigella* isolations per 100 flies (Table 2). About two-thirds of all *Shigella* isolations were made in October, November, and December from approximately one-third of the total flies collected. Samples of flies were taken indoors in the same general area during 9 months of the study. Although a tabulation of these data is not included, three isolations of *Shigella* were made from 2,678 flies caught indoors at Sacaton, a rate of 0.11 isolations per 100 flies, and nine isolations were made from 2,986 flies caught indoors at Guadalupe, a rate of 0.30 isolations per 100 flies. Most of these isolations also were made in October, November, and

TABLE 1

Average grill count of flies, number of outdoor collections, number of flies collected, number of pools examined, and number of Shigella *isolations from Sacaton and Guadalupe, Arizona, May 1954 through December 1955*

Year and month	Sacaton					Guadalupe				
	Ave. grill count of flies	No. flies collected	No. collections	No. pools	No. *Shigella* isolations	Ave. grill count of flies	No. flies collected	No. collections	No. pools	No. *Shigella* isolations
1954										
May	6.0	387	39	44	0	51.9	1,051	65	87	1
June*	3.2	448	39	44	1	25.4	1,531	78	110	2
July	10.6	2,028	87	138	2	50.6	5,253	144	287	0
August*	9.6	1,177	82	105	3	40.1	6,172	159	374	2
September	6.3	911	114	127	2	9.5	2,560	176	236	1
October	4.2	604	112	117	0	18.5	4,434	185	311	3
November*	2.6	432	111	115	0	33.0	5,511	224	387	8
December	1.7	333	83	83	0	16.8	2,691	163	231	13
1955										
January	1.0	118	75	75	0	6.5	787	200	203	0
February	1.5	211	108	108	0	8.5	1,008	257	264	1
March	2.9	359	147	147	0	11.7	1,603	274	285	0
April	4.2	618	142	146	0	14.4	1,475	200	217	0
May	3.4	1,231	153	175	1	20.4	3,110	287	354	3
June	4.2	875	164	172	0	18.9	1,931	243	266	2
July	4.1	688	127	129	0	13.7	1,371	205	211	0
August	13.5	1,427	136	155	1	33.2	4,396	267	359	2
September	11.3	791	149	172	1	10.5	1,137	193	208	0
October*	3.3	604	144	148	4	9.3	1,416	242	260	8
November	3.6	470	134	136	1	20.8	2,045	220	261	4
December	3.5	342	117	119	0	16.5	1,737	188	207	3
Totals		14,054	2,263	2,455	16		51,219	3,970	5,118	53

* Months when *Shigella* isolations from flies exceeded 0.2 per 100 flies.

TABLE 2

Comparison of average number of flies per collection, Shigella *isolations per 100 flies, and percentage of* Musca domestica *from indoor and outdoor collections in Sacaton and Guadalupe, Arizona, May 1954 through December 1955*

Indoor collections			Outdoor collections				
April to December 1955			April to December 1955		May 1954 to December 1955		
Average no. flies per collection	*Shigella* isolations per 100 flies	*M. domestica* % of total flies	Average no. flies per collection	*Shigella* isolations per 100 flies	Average no. flies per collection	*Shigella* isolations per 100 flies	*M. domestica* % of total flies
Sacaton 9.5	0.11	88	5.6	0.1	6.2	0.11	59
Guadalupe 8.7	0.30	88	9.1	0.1	12.9	0.10	70

TABLE 3

Summary of Shigella *isolations by source and* Shigella *serotypes in Sacaton and Guadalupe, Arizona, May 1954 through December 1955*

Shigella serotype	No. of *Shigella* isolations			
	From flies		From human sources	
	Outdoors	Indoors	Rectal swab	Privies
S. dysenteriae 2	1	0	0	1
S. flexneri 1a	3	0	6	0
S. flexneri 2a	0	2	54	6
S. flexneri 3	15	3	38	10
S. flexneri 4a	10	1	46	3
S. flexneri 4b	4	0	12	0
S. flexneri 6	30	4	102	7
S. boydii 2	1	0	13	0
S. boydii 5	0	1	1	0
S. boydii 6	1	0	0	1
S. sonnei 1	4	1	25	0
Totals	69	12	297	28

December when fly population indices in both villages were below average. There were 4 months when the rates of *Shigella* isolations from outdoor flies exceeded 0.2 per 100 flies (Table 1). Three of these occurred within 1 month after high rates of *Shigella* were observed in humans. However, the rates of *Shigella* from flies were generally too low to serve as useful indices of the prevalence of *Shigella* in a community.

Types of Shigella. Eleven different serotypes of *Shigella* were isolated from flies. Of these, 9 types were from flies collected outdoors and 6 types were from flies collected indoors (Table 3). Concurrently with fly sampling, 9 types of *Shigella* were isolated from rectal swab specimens from humans and 6 from fecal samples from privies. The same 11 types were isolated both from flies and from human sources. *S. flexneri* 6 was the most common type isolated from flies and humans. *S. flexneri* 2a was isolated frequently from humans but rarely found in flies. These two types and *S. flexneri* 3, *S. flexneri* 4a, and *S. sonnei* 1 accounted for over 85% of the isolations from both flies and human sources. Some of the rarer types were found as often in flies as from human sources.

Association between Shigella *isolated from flies and human sources.* Of the total of 81 *Shigella* isolations from flies (Table 3), 17 (21%) were from premises where identical *Shigella* types were obtained from human sources within a month. Fourteen of the 17 were from rectal swabs and 3 were from privy samples. Similarly, 44 (54%) of the isolations from flies were collected in the block where the same type was isolated from human sources within a month, 42 from rectal swabs and 2 from privy samples; 68 (84%) were associated by town within a month, 63 from rectal swabs and 5 from privy samples.

Fly populations. Densities of flies were compared by data from fly collections and from grill counts (Table 1). Analysis by seasons showed peaks in both villages during early and late summer. A peak also occurred in Guadalupe during the late fall. The laboratory facilities limited the number of fly pools that could be processed each week. Since the number of pools collected was relatively small, the seasonal variation in fly populations was measured by the number of flies collected rather than by the number of pools or collections. *Shigella* isolations therefore were compared with total flies collected each month for seasonal prevalence.

Outdoor fly populations were considerably higher in Guadalupe than in Sacaton (Tables 1 and 2) and indoor fly populations were slightly higher in Sacaton (Table 2). The house fly, *Musca domestica*, was abundant throughout the year and constituted 70% of all the flies collected in both towns, outdoors and indoors. *M. domestica* was present in 70 (91%) of 77 *Shigella*-positive pools

and 37 (90%) of 41 positive pools that contained only one type of fly. The flies of second importance were *Ophyra spp.*, which also were collected throughout the year but most commonly in the summer. Other genera, in order of prominence, were *Chrysomya*, *Muscina*, *Phaenicia*, and *Sarcophaga*.

Attractants. The major fly attractants in both towns were human excrement, scattered garbage, and waste wash water. Of the 69 isolations of *Shigella* from flies collected out-of-doors, 62 were from these three attractants: 42 from human excrement, 13 from scattered garbage, and 7 from waste wash water.

Size of fly pools in relation to recovery of Shigella. Analysis of *Shigella* isolations by number of flies in each pool indicated that *Shigella* was recovered most frequently in pools of 11 to 15 flies (Table 4). Presumably, fewer flies per pool decreased the chance of including one with *Shigella* and more flies increased the dilution of *Shigella* organisms.

Colony yield on SS plates. During the first 10 months of the study when SS broth was not used, no multiple-colony *Shigella* isolations were obtained. In contrast, when SS broth was employed *Shigella* isolations ranged from one to thousands of colonies. Results for comparable periods when nutrient broth and SS broth were used are presented in Table 5. Although more flies were collected in 1954, the rate of recovery of *Shigella* was higher in 1955, both in total isolations and in isolations per 100 flies. The use of SS broth facilitated recovery of *Shigella* from flies and provided a more sensitive index to the prevalence of *Shigella* in flies.

TABLE 4

Shigella *isolations by size of fly pools in Sacaton and Guadalupe, Arizona, May 1954 through December 1955*

	No. of flies per pool				
	1–5	6–10	11–15	16–20	Over 20
No. pools	3,886	1,002	808	1,155	716
No. *Shigella* isolations	17	8	17	17	10
Isolations per 100 pools	0.44	0.8	2.1	1.5	1.4

TABLE 5

Comparison of nutrient broth and SS broth in recovery of Shigella *from flies*

Medium used	No. collected flies	No. of isolations			
		Single colony	Multiple colonies	Total	Per 100 flies
Nutrient broth, May–Dec. 1954	35,523	38	0	38	0.11
SS broth, May–Dec. 1955	28,708	12	29	41	0.14

DISCUSSION

The methods developed and employed for collecting and processing domestic flies for isolation of *Shigella* were successful. Furthermore, the *Shigella* types from flies were usually the same types isolated concurrently from humans. The general trends of fly populations and of *Shigella* prevalence in or on flies were similar to prevalence of *Shigella* in humans. These results are consistent with those of other studies, *e.g.*, Watt and Lindsay,[3] that showed that flies were involved significantly in the spread of *Shigella*.

Few indications are evident from comparison of types of *Shigella* isolated from flies and from humans. *S. flexneri* 6 was isolated more frequently from both sources. *S. flexneri* 2a was the second most prevalent type from human samples but was isolated only twice from flies. Both isolations were from flies collected indoors in June, 1955. The apparent lack of association between flies and this prevalent type of *Shigella* may be due to low survival ability or high pathogenicity for flies. *S. flexneri* 2a was associated with some of the most severe human cases of shigellosis in the study areas. Most of the other types were recovered too infrequently to permit valid comparison, but it is interesting to note that three types recovered only once from human sources were also recovered from flies.

The fly sampling method may have application in certain situations where high populations of flies occur and a crude index of *Shigella* prevalence must be obtained without examination of persons. A *Shigella* index from flies may also be useful in appraisal of epidemic occurrences.

For example, in an investigation of a diarrheal disease outbreak in a migrant farm labor camp,[8] 297 flies were processed in 40 pools. Isolations of *Shigella* were made from 7 pools, a rate of 2.36 isolations per 100 flies. This rate, about 20 times that of the *Shigella* yield in the endemic areas

considered above, suggested a high fly vectoring potential. *Ophyra spp.* accounted for 44% of the flies collected. All 7 *Shigella*-positive pools contained *Ophyra* and 2 pools were composed of *Ophyra* only.

The period of lowest fly populations and the only period when no *Shigella* isolation was obtained for more than 2 consecutive months was October, 1954, through April, 1955, in Sacaton. The average monthly grill counts ranged from 4.2 to 1.0 during this period. The lowest average monthly grill count when *Shigella* was isolated was 3.2. In Guadalupe, fly counts were higher and isolations of *Shigella* were made more frequently.

Quantitation of transmission rate, or determination of precise relationships between the various factors involved in the spread of *Shigella* by flies, must take into account a multiplicity of variables. These include, in addition to density of flies and prevalence of human infections, exposure of persons and of food to flies, divertive attractants, longevity of flies, climatic and other conditions that affect movement, crowding of housing and people, extent of environmental contamination, and other factors. The influence of some of these factors is illustrated by the partial comparison of Guadalupe and Sacaton.

SUMMARY

Techniques were developed and tested for sampling unbaited domestic flies both outdoors and indoors to determine the natural occurrence of *Shigella* organisms. A total of 65,273 flies collected outdoors yielded 69 isolations of *Shigella* and 5,664 flies collected indoors yielded 12 isolations of *Shigella*.

Comparison of rate of isolations from flies with rate of recovery by the rectal swab technique indicated that the indices from flies generally paralleled the indices from humans, but that they were too low to provide a sufficiently sensitive and effective means of measuring *Shigella* prevalence. Sampling of flies might be used to advantage in certain situations where high fly populations or high *Shigella* prevalence occurs.

Eleven different types of *Shigella* were isolated from flies and the same 11 types were isolated from human sources. Associations in time, location, and *Shigella* type were noted.

Musca domestica was the most abundant species of fly collected and most common in *Shigella*-positive fly pools; *Ophyra spp.* were second in abundance. Major fly attractants were found to be human excrement, scattered garbage, and waste wash water.

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GROWTH OF *COXIELLA BURNETII* IN AGAR TISSUE CULTURE

EDGAR G. PICKENS* AND JAKOB A. GAON†

In 1939 Zinsser *et al.* described a method for growing murine and classical typhus rickettsiae on an agar medium containing minced tissues of guinea pigs, mouse embryos or chicken embryos.[1] The Maitland-type culture was used by Cox and Bell in 1939 for growing *Coxiella burnetii*.[2] In the preparation of an inactivated scrub typhus vaccine, Plotz *et al.* were able to grow *Rickettsia orientalis* on agar tissue culture.[3] Weiss and Pietryk in 1956 grew *C. burnetii* in chick-embryo entodermal cells in monolayer cultures.[4] These techniques were not practical for mass production of rickettsiae in the pre-antibiotic era because of the difficulty in controlling bacterial contamination.

The yolk sac of the embryonated chicken egg has been the medium of choice for producing large quantities of rickettsiae, not only because these organisms grow well in this tissue, but also because bacterial contamination is easily controlled. However, for certain types of research and experimentation, as well as for many practical reasons, a method alternative to yolk-sac culture seemed desirable. In developing such a method, Zinsser's medium, modified by the addition of antibiotics, and a growth medium ordinarily used in monolayer cell cultures were employed for growing *C. burnetii* in a tissue culture system.

MATERIALS AND METHODS

The agar medium used in this work is similar to the one developed by Zinsser *et al.*[1] To 540 ml glass-distilled water, 30 ml Simm's solution "A" and 18 g agar (Difco) were added. This mixture was precooked in flowing steam for 20 minutes and then sterilized at 15 pounds pressure for 20 minutes. After cooling to 50°C the following ingredients were added: 200 ml inactivated serum (equine, bovine or human), 30 ml Simm's solution "B," and 1.6 ml stock Mycostatin® in Hanks' solution (50,000 units/ml). The medium was then dispensed in Blake bottles or 200-ml serum bottles (Kimble Glass Co. Catalogue No. 14250) and incubated at 37°C for 24 hours to check sterility.

LAH-C5 solution, used as a diluent for both rickettsial seed and minced tissue, was prepared according to the following formula. To 100 ml Hanks' solution containing 5% lactalbumin were added 5 ml calf serum, 200,000 units each of penicillin and streptomycin, and 10,000 units of Mycostatin. Sufficient Hanks' solution was then added to make 1 liter.[5]

During preliminary studies, variables which might possibly affect the final yield of *C. burnetii* in tissue culture were explored. First, growth obtained in mouse-embryo tissue was compared with that obtained in chicken-embryo tissue. When the former was used, embryos were taken from females at mid-gestation, the head and feet were removed, and the torsos were minced with scissors and forced through a needleless 50-ml syringe. Chicken embryos 10 to 12 days old were fragmented either by forcing them through a 50-ml syringe or grinding them for 10 seconds in a Waring Blendor.

Two methods of exposing tissue to rickettsial suspensions were compared. Ten to 20 ml of a homogenized yolk-sac culture of *C. burnetii* diluted to varying concentrations in LAH-C5 was mixed with 10 to 20 g minced tissue and incubated for 30 minutes to 1 hour at 4°C. Tissue cells were then separated from the mixture by low-speed centrifugation and planted on agar medium. In the alternate method, 1 ml of inoculum was mixed with 1 to 4 g minced tissue; the mixture was then incubated for 30 minutes to 2 hours at 36°C and planted directly on agar. After inoculation with minced tissue, bottles were closed with rubber stoppers to prevent desiccation.

Other variables tested included size and ratio of inoculum to minced tissue. The effect of the preceding variables on rickettsial proliferation was judged by examining Macchiavello-stained smears of tissue fragments removed periodically during incubation of infected cultures. To

* U. S. Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratory, Hamilton, Montana.

† Department of Epidemiology, Medical School, Sarajevo, Yugoslavia. Participant in a Technical Cooperation Project of the International Cooperation Administration.

obtain more definitive proof that *C. burnetii* had proliferated in tissue culture, the following two studies were conducted.

In the first study, 10 ml of a 1:10 dilution of yolk-sac culture of *C. burnetii*, California strain 76, was added to 10 g of minced chick-embryo tissue. After this mixture was incubated for 1 hour at 36°C it was washed in 10 ml LAH-C5 and the excess fluid was removed by centrifugation at 175 G for 10 minutes. After the washing procedure had been repeated 10 times, sediment remaining after the last washing was planted on 7 bottles of medium, each bottle receiving 1 ml of infected minced tissue. Periodically, during 14 days of incubation at 36°C, the contents of one bottle was removed, suspended in 10 ml sterile skim milk, and ground for 1 minute in a Waring Blendor. This material was then dispensed in glass ampules, shell frozen, and stored on CO_2 ice until the last bottle was processed. Then, the viable rickettsial content of each sample was determined by titrations in which six 21-day-old mice were inoculated intraperitoneally with each decimal dilution. Mice were bled 40 days later and their sera were examined for antibodies against *C. burnetii* by the complement fixation (CF) test and the capillary agglutination (CA) test.[6] Animals possessing CF titers of 1:16 or greater or CA titers of 1:1 (whole serum) or greater were considered to have become infected from the inoculum. Fifty per cent infectious doses (ID_{50}) were determined by the method of Kärber.[7]

In the second study, 8 Blake bottles were each planted with 5 g of chick-embryo tissue infected with 2.5 ml of a 1:20 dilution of a yolk-sac culture of *C. burnetii*, California strain 76. At 3-day intervals after inoculation, the contents of 2 bottles were removed, suspended in 20 ml LAH-C5 and ground for 1 minute in a Waring Blendor. Samples were frozen and stored on CO_2 ice until the growth experiment was completed. The rickettsiae in each sample were then isolated and purified by the method described by Ribi and Hoyer.[8] Briefly, each homogenate was diluted with sufficient LAH-C5 to make 100 ml volume. This was centrifuged for 10 minutes at 400 G in a horizontal rotor and the resulting supernatant was centrifuged for 15 minutes at 1,000 G in an angle rotor to sediment rickettsiae. After resuspending the pellet in M/1 KCl, the crude rickettsial suspension was again centrifuged in an angle rotor at 10,000 G for 15 minutes. Rickettsiae in each sample were then separated from contaminating tissue fragments by controlled centrifugation through a linear sucrose gradient. Suspensions of organisms in the sucrose-KCl mixture were examined in a spectrophotometer. The yield of organisms in each sample was approximated by applying the absorption of ultraviolet light at 260 and 280 mμ to the Warburg-Christian formula,[9] after light scattering at 340 mμ had been subtracted. Electron microscopic examinations of these suspensions were made to determine their purity.

RESULTS

As judged by the examination of Macchiavello-stained smears, growth in mouse-embryo tissue was comparable to that in chick-embryo tissue. The methods of fragmenting tissues, *i.e.*,

TABLE 1

Growth of C. burnetii *in chick-embryo tissue cultures during 14 days of incubation at 36°C as determined by titration in 21-day-old mice*

Culture	Period of incubation	Rickettsiae in smears	Log ID_{50}/g for mice, results of	
			CA test	CF test
1	1 hr	None	6.7	7.5
2	4 hr	None	6.8	7.5
3	8 hr	None	6.1	6.2
4	24 hr	None	6.1	6.8
5	4 days	Very few	7.7	8.7
6	7 days	Very few	6.8	7.5
7	14 days	Large masses	8.7	9.3

TABLE 2

Growth of C. burnetii *in infected chick-embryo tissue cultures incubated for varying periods of time as demonstrated by ultraviolet light absorption measurements and weight of purified organisms*

Period of incubation	Ratio of optical density $\frac{260\ m\mu}{280\ m\mu}$	Optical density (260–340 mμ)	Estimated weight*
None	1:19	0.63	0.27
3 days	1:14	4.62	2.00
6 days	1:16	14.30	6.90
12 days	1:17	15.30	8.00

* Milligrams of purified rickettsiae per 10 g chick-embryo tissue inoculated.

forcing embryos through a syringe or grinding for 10 seconds in a Waring Blendor, did not appear to influence subsequent growth of rickettsiae or viability of tissue cells. Both methods of exposing tissue cells to rickettsiae prior to planting on agar appeared to be equally effective.

In general, the time post-inoculation that rickettsiae were first detected in smears of infected tissue was directly related to the dose. This is illustrated in a titration of growth in an infected tissue culture; rickettsiae were first detected in cultures inoculated with 10^{-2} dilution on the 6th day, those inoculated with 10^{-4} dilution on the 13th day and those inoculated with 10^{-6} dilution on the 24th day post-inoculation. When cultures were inoculated with heavy doses, *i.e.*, 1:10 dilution of yolk-sac culture of *C. burnetii*, maximum growth usually occurred on the 6th to 7th day of incubation. Smears at this time regularly contained more rickettsiae than were ever seen in smears of yolk sacs containing luxuriant growth.

Results of titrations in mice of infected tissue cultures harvested periodically after inoculation showed that growth had occurred (Table 1). During the first 24 hours after inoculation the infectious titer decreased but thereafter it gradually increased. For some reason, growth in culture 6 (7th day) was not as good as in those taken on the 4th or 14th days.

Proliferation of *C. burnetii* in tissue culture was also demonstrated by isolation of the total growth and estimation of its weight by physical means (Table 2). Purified rickettsial suspensions isolated from samples taken 3, 6, and 12 days post-inoculation were free of contaminating elements when examined in the electron microscope (Figure 1). The rickettsial preparation isolated from the unincubated culture represented actual inoculum contaminated with a few nonrickettsial particles. For comparative purposes, the difference in optical density units between the absorption at 260 and 340 mμ of a given sample can be used as a relative measure of the concentration of rickettsiae. These values indicate that the yield had increased 30-fold by the 12th day of incubation and that near maximum growth had been reached by the 6th day of incubation (Table 2).

FIG. 1. Purified *Coxiella burnetii* isolated from infected chick-embryo tissue culture. Magnification 70,000 ×.

DISCUSSION

The results of these experiments indicate that *C. burnetii* proliferated in chick-embryo tissue culture. However, the infectious titer of tissue cultures was considerably lower than that obtained in yolk sacs of embryonated chicken eggs (10^{10} to 10^{11} ID_{50} per g) even though smears of the former always contained more rickettsiae than were found in smears in the latter. Other data indicate that titrations for infectivity do not accurately reflect the total yield of rickettsiae produced in chick-embryo tissue cultures. In other experiments,[8] the yield of purified rickettsiae isolated from 4 different lots of infected chick-embryo tissue culture varied from 0.7 to 1.2 mg per g of original tissue. This yield is comparable to that obtained from yolk sacs of infected embryonated chicken eggs. Furthermore, the CF titer of 20% homogenized suspensions, inactivated by boiling, varied from 1:8 to 1:16 when they were titrated against 16 units of homologous antibody. This antigenic activity is comparable to that of antigens routinely prepared from yolk-sac cultures. These observations indicate that total growth

in minced chicken- or mouse-embryo tissue culture is comparable to that obtained in the yolk sacs of fertile chicken eggs but that the percentage of viable rickettsiae is lower.

Satisfactory vaccines from infected tissue cultures and antigens for the CA test were also prepared. As is commonly recognized, excellent growth is required for both of these products. In the preparation of vaccines, 10% homogenates in saline were inactivated with sufficient formalin to make 0.5% concentration. The vaccine, in rubber-stoppered bottles, was then autoclaved at 15 pounds pressure for 15 minutes. Guinea pigs inoculated with a single 1-ml dose were completely immune when challenged 21 days later with 10^3 minimum infectious guinea pig doses.

Since *C. burnetii* grows equally well in mouse-embryo tissue cultures, this technique would be particularly useful in areas where fertile hens' eggs are not readily available. Because the organism has remained viable in tissue cultures for as long as 110 days, the method provides another means of maintaining strains in areas where storage at freezing temperatures is not possible. Finally, the technique provides another tool whereby basic studies, such as those on phase variation and on morphologic entities, can be investigated.

SUMMARY

Minced chick-embryo tissue, bathed in a lactalbumin growth medium and planted on a modified Zinsser's agar medium, supported excellent growth of *Coxiella burnetii*. Titration of the viable rickettsial content of samples taken during the growth curve indicated proliferation of rickettsiae. Spectrophotometric measurements of purified suspensions of rickettsiae isolated from cultures incubated for varying periods also showed that growth had occurred. Other ancillary observations indicated that the final yield in mouse- or chick-embryo cultures was comparable to that produced in yolk-sac cultures.

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COMPARATIVE ASSAY OF ARTHROPOD-BORNE GROUP C VIRUS ANTIBODIES BY TISSUE CULTURE NEUTRALIZATION AND HEMAGGLUTINATION-INHIBITION TESTS

SONJA M. BUCKLEY AND ROBERT E. SHOPE

The Rockefeller Foundation Virus Laboratories, New York

Since 1954, Causey *et al.*[1] have isolated many arthropod-borne viruses in the Amazon rain forest near Belém, Brazil. Some of these viruses were shown by Casals[2] and by Casals and Whitman[3] to be antigenically related and to constitute a new immunological group which was designated as group C. Viruses assigned to this group have been maintained in the laboratory in suckling mice by intracerebral and peripheral routes of inoculation. Some of the strains have also been propagated[4] in a stable human cell line, strain HeLa (Gey). In this report, attention is especially given to the question whether or not the experimental animal can be replaced by tissue culture methods for demonstration and assay of neutralizing antibodies.

MATERIALS AND METHODS

Viruses. The following virus types were studied: Apeu (An 848), Caraparu (An 3994), Marituba (An 15), Murututucu (An 974) and Oriboca (An 17). Stock suspensions of infected suckling mouse brain, liver or serum (low passage level) were used as source of virus for immunization and adaptation to tissue culture. In addition, stock preparations of each virus were made from pooled fluid phases of infected HeLa cell cultures for use in neutralization testing. Stock preparations were stored for several weeks at −40°C, or for longer periods at −70°C. Storage in the frozen-dried state at −70°C was optimal. Virus titration endpoints were calculated according to the method of Reed and Muench.[5]

Tissue culture techniques. The following stable human cell lines were used: HeLa (Gey) in the original form and in the clonal form as clone S_3 (Puck), $Detroit_6$ (Stulberg), H. Ep. No. 1 (Moore), H. Ep. No. 2 (Moore) and human embryonic intestine (Henle). The cells were grown in 160-ml French square bottles in a growth-promoting medium consisting of 65% Hanks' balanced salt solution, 15% tryptose phosphate broth (Difco) and 20% pooled human adult serum supplemented with antibiotics (100 units of penicillin, 100 μg of streptomycin and 25 μg of Mycostatin® per ml). Before use, the human serum was Seitz filtered; it was not inactivated. The pH of the growth-promoting medium was usually 7.4. The cell inoculum per stock bottle was 10 ml of a cell suspension containing 50,000 cells per ml.[6] The growth-promoting medium, added in amounts of 10 ml per bottle, was changed twice weekly. Cellular transfers were carried out regularly at weekly intervals. Cultures of the slant tube type (16 x 150 mm) were prepared from trypsin dispersed cells (200,000 cells per ml per tube). After an initial growth period of 48 hours at 37°C, monolayers were present. Prior to viral inoculation, the growth-promoting medium was replaced by a maintenance medium (0.9 ml per tube) consisting of 97% Eagle's medium[7]* and 3% calf serum supplemented with the same concentration of antibiotics as described above. The pH of the maintenance medium varied between 6.2 and 6.4. It was adjusted with either sodium bicarbonate (5% stock solution) or Tris (hydroxymethyl) aminomethane[8]† (0.2 molar solution) to render the pH of inoculated cultures approximately 7.2. The pH readings of the growth-promoting and maintenance media were done on a Beckman glass electrode pH meter; the pH of inoculated cultures was determined colorimetrically, with phenol red in the medium, by comparison with color standards. The maintenance medium was changed when indicated, usually 3, 7 and 10 days after inoculation. Cultures were examined at intervals of 2 to 3 days and discarded on the 14th day following the beginning of the experiment.

For maintenance of virus strains, from two to six or more infected cultures and an equal number of uninoculated control cultures were prepared in each passage group. For titration of

* Made up with Hanks' balanced salt solution.

† Tris (hydroxymethyl) aminomethane: Sigma Chemical Co., 3500 DeKalb St., St. Louis, Missouri.

TABLE 1

Immunization schedule

Animal	Virus type*	Source of virus	Vaccine	Amount inoc. (ml)	Route of inoc.	Time interval (days)
Mouse	Caraparu Marituba Murutucu Oriboca	Liver	Inact.	0.2	i.p.	0 time
Mouse	Caraparu Marituba Murutucu Oriboca	Liver	Inact.	0.2	i.p.	3
Mouse	Caraparu Marituba Murutucu Oriboca	Liver	Live	0.2	i.p.	10
Mouse	Apeu	Brain	Live	0.03	i.c.	0 time
Rabbit	Apeu Caraparu Marituba Murutucu Oriboca	Liver	Live	2.0	i.p.	0 time
Rabbit	Apeu Caraparu Marituba Murutucu Oriboca	Liver	Live	2.0	i.p.	35

* One virus type used to inoculate each animal.

TABLE 2

Titrations of stock preparations of group C viruses propagated in HeLa cells

Virus strain	Passage level	LD_{50} /1 ml	TCD_{50}*/1 ml
Apeu	8	5.2	4.3
Caraparu	8	3.0	3.6
Marituba	12	5.0	4.8
Murutucu	12	5.6	5.9
Oriboca	12	7.5	7.6

* TCD_{50} titers: neg. $\log_{10}$ per 1 ml of infected fluid phase of tissue culture.

viruses, usually six to twelve, but occasionally three, tubes were used per dilution of virus, the number varying with the purpose of the test.

For histopathologic studies, cells were grown on coverglasses placed in Leighton tubes.* Bouin's fluid was used as fixative, and the fixed tissues were stained by Giemsa's method.

Sera. Immune sera were prepared in 6- to 8-week-old mice and adult male rabbits according to the immunization schedule shown in Table 1.

Preparation of vaccines. Livers or brains of infected suckling mice were homogenized and suspended 1 in 10 by weight in borate saline, pH 9.0.[9] In order to destroy infectivity[10, 11] some of the preparations were incubated for 1 hour at 37°C with 0.2% beta-propiolactone.†

Mice were bled on the days indicated in Table 4. Rabbits were bled at weekly intervals and again 14 days following the administration of a booster dose inoculated intraperitoneally about 5 weeks following the first immunizing dose. Blood specimens from uninoculated mice and rabbits served as controls.

Immune mouse and rabbit sera, as well as pooled sera of uninoculated mice and rabbits, were stored at −20°C or at −70°C.

Tissue culture neutralization tests. All tests were carried out in HeLa cells. The constant virus-varying serum method was used. Table 2 lists TCD_{50} and LD_{50} titers (neg. $\log_{10}$ per 1 ml) of the stock preparations employed in these experiments. The viruses were thawed freshly for each test. Test sera, inactivated for 30 minutes at 56°C, were diluted in 2-fold steps in phosphate buffered saline, pH 7.2, containing 0.75% of bovine albumin (Armour fraction V). The starting dilution was 1:10. Each serum dilution was mixed with approximately 100 TCD_{50} doses of virus, incubated at 37°C for 1 hour and inoculated into three HeLa cultures. With each series of neutralization tests, control titrations of viruses were carried out, the starting concentration being the highest dose (100 TCD_{50}) used in the serum-virus mixtures. The highest concentration of each serum was also tested in tissue culture for any possible nonspecific degeneration. In each test series, at least two cultures were left uninoculated to serve as cell control. In evaluating the data, tubes showing complete destruction of the monolayer (4+), or involvement of most of the cells with detachment of many of them from the tube wall (3+), were included in the endpoint calculations, whereas tubes showing only a partial cytopathogenic effect (2+), or no lesions (0), were excluded. The titer of a serum was expressed as the highest serial 2-fold dilution

* Leighton tubes: Microbiological Associates, Bethesda, Maryland.

† Betaprone®: Testegar and Co., Detroit, Michigan.

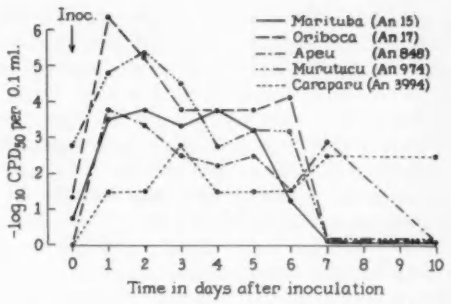

FIG. 1. Growth curves of group C viruses in HeLa TPB cells.

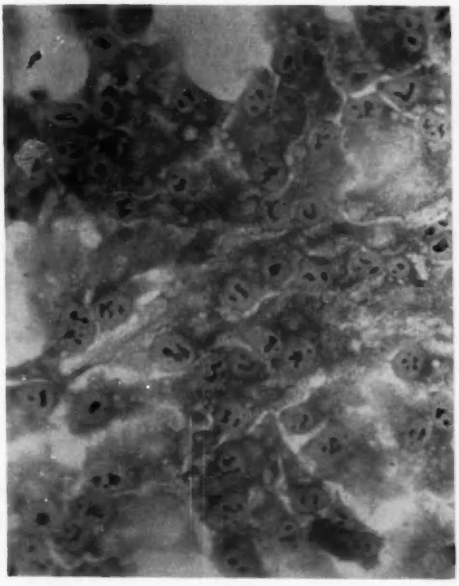

FIG. 2. HeLa TPB cells, uninoculated, stained by Giemsa's method. Monolayer intact. ×570.

of serum that prevented the development of lesions in three out of three or at least in two out of three cultures within an observation period of 14 days.

HI tests. The HI test was the standard against which the specificity of the tissue culture neutralization test was evaluated. Hemagglutinating (HA) antigens were prepared from suckling mouse sera by methods described by Clarke and Casals.[9]

Test sera were diluted in 2-fold steps, the starting dilution being 1:10. Each serum sample

FIG. 3. HeLa TPB cells inoculated with Oriboca virus and harvested 72 hours after inoculation; stained by Giemsa's method. Monolayer destroyed. ×570.

was tested against five HA antigens prepared from virus strains Apeu, Caraparu, Marituba, Murutucu and Oriboca.

RESULTS

Adaptation of Viruses to Tissue Culture

HeLa cells. Inocula of large size were used to initiate passages. Cytopathogenic effect (CPE) was present in the first passage with all five virus types.* Low passage level cultures were successfully established with the undiluted fluid phase of cultures showing clearly distinguishable, but not too far advanced, CPE. The different growth curves of the five virus types obtained during early passage levels following inoculation of a large virus dose are shown in Figure 1. Tissue culture cytopathogenic doses$_{50}$ (TCD$_{50}$) were based on destruction of monolayers. During later passages, the viruses were maintained easily by serial passage of diluted culture fluids. During the period of viral synthesis, fluids of infected

* During preparation of this manuscript, Itaqui virus, a sixth prototype of group C, was propagated in HeLa cells; CPE was regularly observed.

cultures became more acid than those of uninoculated cultures. The initial cytopathogenic lesion, which appeared within 2 to 5 days, was focal in nature and made up of small groups of rounded and granulated cells. Such cells tended to detach from the tube wall. Other foci were soon visible within the cell sheet and these coalesced to produce ultimate destruction of the entire monolayer (Figures 2, 3). With Oriboca virus, severe destruction of cell sheets occurred within 5 days following inoculation of a small virus dose. With the other four virus types, monolayers were destroyed within 7 days. Specific antisera prevented the development of CPE.

Other human cell lines. Five additional stable cell lines were then tested for their ability to support growth in vitro of the five virus types of group C. Source of virus (see Table 2) was tissue culture fluid of stock preparations (stored in the wet state) of infected HeLa cells. Titrations of the virus inoculum, rather than serial passages,

TABLE 3

Growth in vitro of group C viruses in stable human cell lines

Virus inoculum			TCD_{50} titers* in different cells				
Strain	Number of passages in HeLa cells	TCD_{50}*	HeLa, clone S_2	Detroit$_6$	H. Ep. No. 1	H. Ep. No. 2	Human embr. intestine
Apeu	8	4.3	3.5	3.0	4.3	4.4	4.4
Caraparu	8	3.6	2.5	2.5	2.5	2.5	3.0
Marituba	12	4.8	3.5	5.0	5.2	5.0	5.5
Murutucu	12	5.9	5.5	6.0	6.4	5.5	5.7
Oriboca	12	7.6	7.2	7.1	7.2	7.4	7.0

* TCD_{50} titers: neg. $\log_{10}$ per 1 ml of infected fluid phase of tissue culture.

TABLE 4

Hemagglutination-inhibition tests on immune mouse sera (4 units)

Serum	Bleeding day after live vaccine	Antigen				
		Apeu	Caraparu	Marituba	Murutucu	Oriboca
Apeu	18, 21 pooled	640*	80	0	0	0
Caraparu	17	320	2560	80	80	40
	19	320	1280	80	80	20
	28	320	1280	160	80	20
Marituba	9	0	10	160	40	0
	14	20	40	320	80	0
	20	20	40	320	80	0
	27	20	20	160	80	10
Murutucu	14	80	160	320	1280	20
	20	80	80	320	640	10
	27	80	160	320	640	10
Oriboca	17	10	0	10	0	320
	19	20	20	10	10	320
	28	10	0	10	0	640

* 640 = reciprocal of highest serum dilution inhibiting hemagglutination. Starting dilution = 1:10; 0 signifies less than 1:10.

were carried out in these experiments. The results are summarized in Table 3. Again, TCD_{50} titers were based on complete destruction of monolayers. In all instances, progressive CPE, similar to that described for HeLa cells, was observed. Further propagation of the viruses in these human cell lines was not attempted.

In all these experiments, cells of stable human cell lines were cultivated in a growth-promoting medium which contained 15% tryptose phosphate broth. During viral synthesis, cultures were maintained in Eagle's medium supplemented with calf serum. This combination of growth-promoting and maintenance media provided conditions which were suitable for the propagation of group C viruses that caused destruction of the human cells. In addition, a hydrogen-ion concentration (pH) of 7.2 ± 0.2 during adsorption of viruses to cells and during the viral growth period produced optimal and reproducible TCD_{50} titers. The pH values of 7.5 or 6.9 at the time of viral inoculation resulted in a delayed appearance of CPE and a loss in virus titers of up to 1 $\log_{10}$. When virus titrations were carried out in a cell system maintained in a medium buffered with either sodium bicarbonate or Tris (hydroxymethyl) aminomethane[7] to a pH of 7.6 to 7.8 at the time of inoculation, there was a loss in virus titer of up to 2 $\log_{10}$s. In such a highly buffered system, tubes inoculated with a small virus dose exhibited a more rapid and complete CPE than did cultures inoculated with a large virus dose. Eggers[12] made similar observations in monkey kidney cultures inoculated with Marituba and Oriboca viruses. In his experience, a pH of 7.6 to 7.8 during the period of viral multiplication delayed the CPE, which was often incomplete. However, upon treatment of such cultures with CO_2 prior to viral inoculation, onset of CPE became more rapid and destruction of monkey kidney cells complete. Enders[13] has stressed the fact that the pH of the maintenance medium should be kept as nearly as possible within physiological limits during the cultivation of mammalian viruses.

Tests with Immune Mouse Sera

HI tests. Antibodies were demonstrated in all instances within 9 to 18 days after inoculation of live vaccine. With the homologous antigens (see Table 4), titers of sera were one or more dilutions higher than with the heterologous antigens. Oriboca virus showed only an insignificant degree of cross-reaction with the other four virus types, whereas Apeu and Caraparu, on the one hand, and Marituba and Murutucu, on the other, were closely related. There was also a considerable amount of cross-reactivity between Caraparu, Marituba and Murutucu, and between Murutucu and Apeu. Fewer cross-reactions were encountered in the case of Apeu. These observations confirm studies by Casals and Whitman.[3]

TABLE 5

Hemagglutination-inhibition (4 units) and neutralization tests on immune mouse sera (homologous system)

Serum	Bleeding day after live vaccine	HI	NT	
		Titer	Titer	TCD_{50}
Apeu	18, 21 pooled	640*	160*	1.7†
	18, 21 pooled	640	20	3.5
Caraparu	17	2560	160	1.5
	17	2560	80	2.1
	19	1280	160	1.5
	28	1280	160	1.5
Marituba	14	320	20	2.5
	20	320	20	2.5
	27	160	10	2.5
Murutucu	14	1280	80	1.5
	14	1280	40	2.8
	20	640	80	1.5
	27	640	40	1.5
Oriboca	17	320	160	1.0
	17	320	40	2.2
	19	320	160	1.0
	28	640	160	1.0

* 640 or 160 = reciprocal of highest serum dilution inhibiting hemagglutination or preventing development of CPE.

† $TCD_{50}(\log_{10})$ used in neutralization test.

Neutralization tests. Homologous systems were tested (see Table 5). For convenience, HI titers are included in the table. There was no gross discrepancy between results obtained in vitro by the two methods. Although it was found that considerable variations in the amount of virus influenced only moderately the titer of a serum, correlation between HI and neutralization tests was better when the TCD_{50} doses were less than

100. As shown in Table 5, a decrease from 3.5 to 1.7 TCD_{50} doses (log_{10}) resulted in an increase in serum titer from 1:20 to 1:160. With Caraparu virus, a decrease from 2.1 to 1.5 TCD_{50} doses (log_{10}) caused a rise in titer from 1:80 to 1:160. Similarly, in the case of Murutucu virus, a reduction from 2.8 to 1.5 TCD_{50} doses (log_{10}) resulted in a titer increase from 1:40 to 1:80. With Oriboca virus, the titer was increased from 1:40 to 1:160 when the TCD_{50} doses were decreased from 2.2 to 1.0 (log_{10}). Considering the intrinsic difference in the antigens used in

TABLE 6

Hemagglutination-inhibition tests on immune rabbit sera (4 units)

Serum	Bleeding day after first dose of live vaccine	Antigen				
		Apeu	Caraparu	Marituba	Murutucu	Oriboca
Apeu	Pre*	0†	0	0	0	0
	7	0	0	0	0	0
	35	0	0	0	0	0
	49	320	80	0	0	0
Caraparu Rabbit #1	Pre	0	0	0	0	0
	7	80	160	20	20	0
	16	40	160	20	10	0
	29	80	160	20	20	10
	35	80	160	20	20	10
Caraparu Rabbit #2	Pre	0	0	0	0	0
	7	0	10	0	0	0
	35	0	10	0	0	0
	49	20	80	20	20	0
Marituba Rabbit #1	Pre	0	0	0	0	0
	7	0	0	0	0	0
	35	0	0	0	0	0
	49	0	10	40	10	0
Marituba Rabbit #2	Pre	0	0	0	0	0
	7	0	0	40	10	0
	13	0	0	80	20	0
	21	0	0	160	40	0
	28	0	10	320	40	0
	35	0	10	320	40	0
	49	10	10	320	40	0
Murutucu	Pre	10	10	10	10	10
	7	0	0	0	10	0
	35	10	0	10	10	10
	49	40	80	320	640	10
Oriboca	Pre	0	0	0	0	0
	7	0	0	0	0	40
	16	0	0	10	0	80
	29	0	0	0	0	80
	35	0	0	10	0	80
	49	0	0	10	0	320

* Pre: pre-inoculation bleeding obtained from each rabbit prior to first inoculation of live vaccine.

† Results are expressed as the reciprocal of the highest serum dilution inhibiting hemagglutination. Starting dilution = 1:10; 0 signifies less than 1:10.

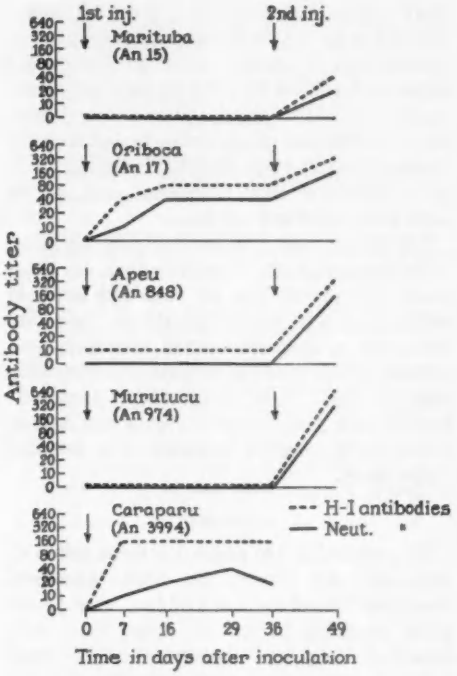

FIG. 4. Development of HI antibodies and neutralizing antibodies in rabbits.

HI and neutralization tests, it would appear that the 100 TCD_{50} doses commonly applied in neutralization tests represent a larger amount of antigen than the 4 units of HA antigen incorporated in HI tests. Control sera did not prevent the development of CPE.

Tests with Immune Rabbit Sera

HI tests. Results of these tests are summarized in Table 6. In every instance, homologous titers of serum samples were higher than the heterologous titers, showing a difference of at least one 2-fold dilution. Again, Apeu and Caraparu viruses, and Marituba and Murutucu viruses, respectively, were closely related. Individual rabbits varied greatly in their antibody response. Some of them showed a development of antibodies 1 week following the administration of virus, whereas others reacted only after the inoculation of a booster dose.

Neutralization tests. Figure 4 shows the development of antibodies in some of the rabbits. Whenever HI antibodies were detected, neutralizing antibodies were present also.

In one series of experiments (see Table 7), serum samples from immune rabbits bled 49 days after the first administration of virus and again 14 days following the booster dose were

TABLE 7

*Cross hemagglutination-inhibition (4 units) and neutralization tests on immune rabbit sera**

Serum	Antigen														
	Apeu			Caraparu			Marituba			Murutucu			Oriboca		
	HI	NT		HI	NT		HI	NT		HI	NT		HI	NT	
	Titer	Titer	TCD_{50}‡	Titer	Titer	TCD_{50}	Titer	Titer	TCD_{50}	Titer	Titer	TCD_{50}	Titer	Titer	TCD_{50}
Apeu	*320*†	*160*†	*1.7*	80	10	2.5	<10	10°	3.5	<10	0§	3.2	<10	0	2.5
Caraparu Rabbit #2	20	10	2.7	*80*	*160*	*0.7*	20	0	2.5	20	2	2.5	<10	0	2.5
Marituba Rabbit #2	10	10°	2.5	10	2	3.2	*320*	*80*	*3.5*	40	20	2.2	<10	0	2.7
Murutucu	40	10°	3.5	80	10	3.5	320	10	4.5	*640*	*80*	*2.5*	10	0	3.0
Oriboca	<10	0	4.5	<10	0	2.7	10	0	3.5	<10	0	2.5	*320*	*80*	*2.2*

* Rabbits bled 49 days after first dose of live vaccine.

† 320 or 160 = reciprocal of highest serum dilution inhibiting hemagglutination or preventing development of CPE.

‡ TCD_{50} (log_{10}) used in neutralization test.

§ 0 = undiluted serum (10°) did not prevent the development of CPE.

tested against the five virus types in cross-neutralization tests. In each instance, homologous titers were higher than the heterologous titers. Oriboca virus did not show any cross-reactions, whereas Apeu and Caraparu as well as Marituba and Murutucu were closely related. In addition, there was some cross-reactivity between Murutucu and Caraparu, which was present also in the HI test. Control sera did not prevent the development of CPE.

DISCUSSION

For the neutralization test in tissue culture, it is essential that the representative strains produce a sharp, reproducible CPE. In this investigation, a well defined combination of growth-promoting and maintenance media, together with careful attention to the pH conditions during the periods of viral adsorption to the cells and viral synthesis, assured rapid CPE and accuracy of test series. Since it was desirable to examine a range of serum dilutions tested also by HI, the constant virus-varying serum technique was chosen. This method also was favored because virus preparations with low titers can be used, and because toxicity of test sera is eliminated through dilution. Tyrrell and Horsfall,[14] while studying different virus-host systems, have pointed out other advantages of the constant virus-varying serum technique over the constant serum-varying virus technique.

In tests of homologous systems, the correlation between the two in vitro methods, one measuring HI antibodies and the other measuring neutralizing antibodies, was very good. In 51 homologous neutralization tests on immune mouse and rabbit sera (Tables 5 and 7; Figure 4), presence or absence of neutralizing antibodies correlated accurately with presence or absence of HI antibodies when the same serum samples were examined by HI tests.

In the past, neutralizing antibodies against group C viruses have been detected and assayed in mice.[1, 3] Since the viruses show a variable pathogenicity for adult mice but are highly pathogenic for suckling mice by both intracerebral and peripheral routes of inoculation, the suckling mouse represents the optimal in vivo host system for the assay of neutralizing antibodies. During the last decade, however, it has been demonstrated for many viruses that resort to the living animal is unnecessary, if viral cytopathogenic changes produced within cultured cells can be adequately prevented by the addition of specific antibody. The present investigation shows that the experimental animal can be replaced by tissue culture in the demonstration and assay of antibodies against group C viruses. In comparison with in vivo tests, the in vitro method is more economical and, at the same time, rapid and accurate.

The HeLa strain of human malignant epithelial cells, employed more extensively than any other stable human cell line for viral and antibody assays, has been chosen for the in vitro neutralization studies. Stable cell strains in continuous culture have the advantage over primary cultures that their morphology, metabolic activity and rate of multiplication can be stabilized during regular transfers in a constant environment.

SUMMARY

Five viruses of the arthropod-borne group C, designated by Causey as Apeu, Caraparu, Marituba, Murutucu and Oriboca, were propagated in stable human cell lines. Each virus caused a sharp and reproducible CPE when cells were grown in a growth-promoting medium containing tryptose phosphate broth and maintained during the period of viral synthesis in Eagle's medium supplemented with calf serum at a pH of 7.2 ± 0.2.

Results of parallel HI and neutralization tests on immune mouse and rabbit sera, using a HeLa cell-virus system and a constant virus-varying serum technique for the determination of neutralizing antibodies, showed no discrepancies between the two in vitro methods when homologous systems were tested.

The experimental evidence indicates that the tissue culture neutralization test may be substituted for the mouse neutralization test or the HI test in immunological studies of the arthropod-borne group C viruses.

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ECHO VIRUS TYPE 13

II. Epidemiologic Aspects and Clinical Associations*

WILLIAM McD. HAMMON, DAVID S. YOHN, ROSE A. PAVIA AND GLADYS E. SATHER

Department of Epidemiology and Microbiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania

Previously we described[1] the characteristics of two strains of ECHO-13 virus which had been isolated from two normal persons living in the Philippines during a longitudinal study of poliovirus infections at Clark Air Force Base (CAFB) in 1953.[2] The selection of strain Del Carmen 11-4(pl) as prototype to replace the original strain Hamphill 2-188-20 was also discussed previously.[1] This report describes available clinical and epidemiologic data on laboratory confirmed human infections with ECHO-13 virus. The only previous pertinent and published data regarding central nervous system (CNS) damage possibly produced by this virus are a case of human aseptic meningitis[3] and a brief description of neuronal pathology in monkeys inoculated by the intramuscular route.[4]

METHODS

The laboratory methods employed have been described previously[2] with the exception of the preparation of ECHO virus complement-fixing (CF) antigens. These were prepared from infected trypsinized rhesus monkey kidney cell cultures (RKMKCC) which were frozen and thawed, centrifuged at 2,000 rpm for 20 minutes and the supernatant stored in aliquots at −20° to −30°C. Just prior to use they were heated for 20 minutes at 56°C and used undiluted.

VIRUS ISOLATION

ECHO-13 virus was isolated from rectal swabs of 10 apparently healthy children in the CAFB area of Luzon, all of whom were participants in the aforementioned survey.[2] The ages were 4, 7, 11, 12 and 20 months (1 child each) and 2 years (5 children). One specimen contained both ECHO-12 and 13 viruses while another, Hamphill 2-188-20, described previously,[1] probably contained a mixture of ECHO-1 and 13 viruses. A throat swab of one of these infants also yielded ECHO-13 virus.

SEROLOGIC RESPONSE AND EVIDENCE OF INFECTION

Hamphill family. The results of serologic tests on selected specimens from the Hamphill family, which included infant R.W., from whom the original ECHO-13 prototype virus was isolated, are shown in Table 1. ECHO-13 neutralizing antibody (NA) titers for this infant were not determined since no serum remained after several previous NA and CF antibody tests had been made for poliomyelitis and certain other ECHO viruses. NA rises to ECHO-13 virus were demonstrated in a 4-year-old sibling (R.A.) and in the father (R.D.), age 27. These titers rose from less than 1:4 to 1:16 and from less than 1:4 to 1:32, respectively, within an 8-week period. In both instances, the maximum observed titer was attained 4 weeks before the virus was isolated from the infant member of the family. In the father, the ECHO-13 virus NA titer fell from 1:32 to 1:8, 4 weeks after the higher titer was obtained.

The infant (R.W.) showed CF antibody rises to poliovirus type III and to ECHO viruses types 1 and 13 from less than 1:4 to 1:8, less than 1:4 to 1:32 and less than 1:4 to 1:32, respectively. These three viruses were isolated from this infant at different times within a period of less than 3 months (Table 1). The CF antibody response to ECHO-13 virus preceded the isolation of this virus by 8 weeks, indicating that infection probably occurred sooner than detected by virus isolation. Both sibling (R.A.) and father (R.D.)

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Grateful acknowledgment of the technical assistance of Miss Della M. Whitaker is made.

TABLE 1

Results of laboratory tests on selected specimens from the Hamphill family

Family member	Age	Specimen	Date	Virus isolation	Neutralizing antibody				CF* antibody				
					ECHO 13	Poliovirus			ECHO		Poliovirus		
						I	II	III	13	1	I	II	III
R.W.	20 mo.	Serum	8/10		—†	<4	<4	<4	<4	<4	<4	<4	<4
		Throat swab	9/7	E-1‡									
		Serum	10/5		—	—	—	4	32	32	<4	<4	<4
		Rectal swab	11/2	P-III§									
		Serum	11/30		—	4	<4	1,024	32	32	<4	<4	8
		Rectal swab	11/30	E-13									
R.A.	4 yr.	Serum	8/10		—	<4	<4	<4	—	—	<4	<4	<4
		Serum	9/7		<4	—	—	<4	—	—	<4	<4	<4
		Throat swab	9/21	E-1									
		Serum	10/5		8	—	—	8	—	—	<4	<4	<4
		Rectal swab	10/5	P-III									
		Serum	11/2		16	<4	<4	256	—	—	<4	<4	8
		Serum	11/30		—	<4	<4	256	—	—	<4	<4	16
R.D.	27 yr.	Serum	9/7		<4	64¶	64¶	<4	—	—	4	4	<4
		Serum	10/5		—	64	256	<4	—	—	4	4	<4
		Rectal swab	10/5	P-III									
		Serum	11/2		32	1,024	>1,024	512	—	—	16	16	8
		Serum	11/30		8	512	>1,024	512	—	—	8	32	8

* CF = complement fixing.
† — = not done.
‡ E = ECHO.
§ P = poliovirus.
¶ Test actually performed on specimen collected 1 month earlier.

TABLE 2

Results of serologic tests on patients R.D. and M.J.

Patient	Days after onset	Neutralizing antibody							CF* antibody			Jap B HAI†
		ECHO			Poliovirus			Cox A-9	Poliovirus			
		13	1	12	I	II	III		I	II	III	
R.D.	4	<4	<4	32	64	4	1,024	128	8	32	32	
	18	8	<4	16	64	4	1,024	64	16	32	64	Neg.
M.J.	Before‡	<4	<4	<4	<16	<16	64	64	<4	<4	<4	
	3	8	<4	<4	<4	<4	64	<4	<4	8	4	
	16	32	<4	<4	<4	<4	64	<4	<4	8	<4	
	42	8	8	<4	<4	<4	64	<4	<4	16	<4	

* CF = complement fixing.
† HAI = hemagglutination inhibition.
‡ Serum drawn 1 year before onset. Test results shown for this specimen were obtained at an earlier time than were those on the subsequent specimens.

were also infected with poliovirus type III as demonstrated by virus isolation; in addition, R.A. was similarly shown to have been infected with ECHO virus type 1. No illness occurred in any member of this family in association with any of these three different viral infections.

Other infected persons. No sera were collected from the Del Carmen infant from whom 11-4(pl), the new ECHO-13 prototype strain, was isolated.[1] Similarly, no sera were available from the 8 other persons from whom ECHO-13 virus was isolated.

Serologic evidence only of past or current infection with ECHO-13 has been obtained from 9 other persons, in addition to the 2 older members of the Hamphill family. These 9 came from a group of 32 persons, 23 of whom were between the ages of 19 and 38 years, and 9 below that age. These persons were all American patients hospitalized at various times at CAFB with a CNS disease. All but two, it was eventually shown, had illnesses not attributable to ECHO-13 virus. From this group of 32 patients, eight aged 21 to 29 years and one 11-year-old were shown to have NA titers ranging from 1:4 to 1:64, indicating previous or current infection with ECHO-13 virus.

CLINICAL FINDINGS

During the course of their illnesses, two patients among the above-selected group showed a significant NA rise to ECHO-13 virus and not to polioviruses nor to several other enteroviruses included in the battery of tests (Table 2). A brief summary of these two cases follows:

Patient R.D., a male airman, age 21 years, developed a classical clinical picture of mild paralytic poliomyelitis. Weakness of the neck and abdominal muscles was still present on the 25th day; consequently he was promptly evacuated to the United States. Blood sera were taken on the 4th and 18th days after onset. The results of serologic tests are shown in Table 2. No virus was isolated from a fecal specimen taken on the 3rd day, but the specimen had been lost in transit for a long period and refrigeration during this time was of an uncertain nature. The only significant serologic rise observed was to ECHO-13 virus.

Patient M.J., a female dependent, age 12 years, had a mild febrile, aseptic meningitis syndrome. Results of serologic tests on four specimens, the first taken by us during the poliomyelitis survey[2] a year before the onset of illness, are shown in Table 2. A rise in NA titer to ECHO-13 virus from 1:8 to 1:32 occurred during the acute phase of the illness. No antibody was present the year before. This represents the only NA rise demonstrated during the acute phase of the illness. The slight NA rise to ECHO-1 virus between the 16th and 42nd day probably represents a later infection. The elevated poliovirus type II CF antibody, in the absence of detectable NA to the same virus, is interpreted as evidence supporting the concept of a common antigen among certain enteroviruses which has been noted in many instances.[3] This latter antibody might have been acquired any time during the interval of 1 year which elapsed between the collection of the survey specimen and collection of the first of the three clinical specimens (Table 2). No virus was isolated from a specimen of feces taken on the 2nd day after onset, again probably because of the delay in shipment.

INOCULATION OF MONKEYS

Various strains of ECHO-13 virus were inoculated into 16 monkeys by either the intracerebral, the intraspinal, the intravenous, or the intramuscular route or combinations of these routes. No signs of illness developed. However, chronic meningoencephalitis of the brain stem and midbrain but not of the spinal cord was observed in sections taken from two monkeys which had been inoculated by both the intracerebral and intraspinal routes and then sacrificed 21 days after inoculation.

GEOGRAPHIC DISTRIBUTION

Information regarding the geographic distribution of this virus is limited. In addition to our previously reported isolations made in the Philippines,[1] Rosen *et al.*[5] reported the isolation of a virus called JV-2 from nursery children in a Washington, D. C., welfare institution. This agent in our laboratory, and subsequently in that of Rosen, was typed as an ECHO-13 virus.

More recently Rosen *et al.*[6] reported 38 isolations of ECHO-13 from October, 1955, to February, 1956, from this institutionalized population. In no instance was a conclusive causal relationship made between ECHO-13 infection and clinical illness even though onsets of illnesses characterized by coryza, pharyngitis, or fever,

singly or in combination, were noted concurrent with initial virus shedding.[6]

Cheever and Abraham[7] in Pittsburgh, while studying two small outbreaks of febrile diarrhea in a juvenile court institution in September and December of 1959, obtained 4 isolations of ECHO-13 virus from single fecal specimens taken from 28 children. Several others showed antibody rises for ECHO-13 virus from less than 1:8 in the acute phase serum, to 1:32 up to 1:256 in the convalescent specimens. Sixteen other agents identified as belonging either to the enterovirus or adenovirus groups, but not ECHO-13, were isolated at the same time from other children in the groups.

A single lot of Japanese gamma globulin was found to contain NA to ECHO-13 virus at a 1:50 dilution; thus the virus is probably present in Japan. A single lot of American gamma globulin did not neutralize either the 2-188-20 or the 11-4(pl) strain at a 1:25 dilution; this was the lowest dilution found to be non-toxic for MKCC. Three pools of four sera each, made from specimens collected from adult Filipinos, neutralized both of the above strains at 1:5 to 1:10 dilutions. There is some evidence to indicate that both CF antibodies and NA's do not remain elevated for long periods after infection;[1,3] thus negative results with tests on gamma globulin and in serologic surveys may not have the same significance in studying geographic distribution as for many other viruses.

SUMMARY AND CONCLUSIONS

ECHO-13 virus was first obtained from rectal and throat swabs collected in the Philippine Islands in 1953. Isolations were made from 10 apparently healthy children. Serologic evidence of infection was obtained in 11 other individuals.

ECHO-13 virus, although not neutralized at a 1:25 dilution by a single pool of American gamma globulin, has been isolated recently in two cities of the United States. Since it is neutralized by Japanese gamma globulin, it is probably also present in Japan. Apparently both complement-fixing and neutralizing antibodies do not remain elevated for long periods of time after infection.[1,3] This limits the usefulness of serologic surveys in quantitative studies of the geographic distribution of ECHO-13 virus and the significance of absence of detectable antibody in gamma globulin as a qualitative test for absence of the virus in a population.

Two patients hospitalized with CNS disease, one of whom was paralyzed, showed a significant serologic response only to type 13 ECHO virus during the course of their illnesses. CNS pathology was observed in two inoculated monkeys. These data, which may be interpreted as presumptive evidence that ECHO-13 may produce CNS disease in man, are too limited and incomplete to permit any definite conclusions to be drawn at this time; but the increasing evidence that a number of the ECHO viruses and the enteroviruses as a group do possess the potential of producing CNS disease[3, 4, 8-13] lends more weight to the probability that an occasional human CNS illness is attributable to ECHO-13 virus. Isolations and serological rises from a few children during outbreaks of diarrhea in Pittsburgh also do not lend themselves to interpretation since several other viral agents were isolated from the feces of other patients. This report therefore can serve only to point suspicion to ECHO-13 virus as a pathogenic agent. It shows what may be expected in terms of virus isolation and serological responses during infection, spread within a family, some of the limitations of serologic survey and summarizes what is known regarding geographic and seasonal distribution.

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TWO NONFATAL HUMAN INFECTIONS WITH THE VIRUS OF EASTERN ENCEPHALITIS

DELPHINE H. CLARKE

The Rockefeller Foundation Virus Laboratories, New York

In the late summer and fall of 1959, an outbreak of Eastern viral encephalitis (EEE) occurred in southern New Jersey during the course of which there were 29 confirmed cases in man, with 20 fatalities.[1] While the epidemic was in progress, a laboratory investigator (R.S.) became infected with the virus of EEE, either as a consequence of visiting the infected area or through laboratory contact with the virus. The present communication describes his illness, as well as another nonfatal infection possibly occurring at the same time, and the associated laboratory studies.

CLINICAL PICTURE

The following descriptions are based, in the main, on personal recollections since physicians were not consulted during the acute phase of the disease. The onset of illness of R.S. was abrupt, with presenting symptoms of malaise, arthralgia and myalgia. Within a few hours, chilly sensations were experienced, followed shortly by severe shaking chills which persisted throughout the night. The picture remained more or less unchanged over the next 3 days, with repeated bouts of chills at night. An oral temperature of 104°F was recorded on the afternoon of the 2nd day and 100°F on the 3rd. On the 5th day, some symptomatic improvement was noted, but on the 6th day the symptoms recurred and persisted, with a return of shaking chills on the 8th day. Chilliness, malaise and generalized aches and pains continued for 2 more days. By the 11th day definite improvement was noted, and from then on no further discomfort was experienced. At no time was there headache or any other symptom referable to the central nervous system. There was no nausea nor anorexia. Recovery was complete, with no sequelae. Three and a half months after onset, R.S. was given a complete neurological examination at New York Hospital by Dr. Charles E. Wells. No abnormalities of any sort were found with the exception of occasional, paroxysmal, irregular activity in the electroencephalogram, mainly in the temporal areas. This was interpreted as representing a pathological pattern *possibly* the result of the EEE infection but nonspecific in nature and similar to changes seen in many processes.

Another probable mild case of EEE virus infection occurred in P.G., a man who lived and carried on outdoor work in the same area of New Jersey where R.S. had his possible exposure. The pertinent history here was the sudden onset of a stinging sensation in the right flank 3 days prior to the onset of the illness of R.S. This sensation spread to immediately adjacent areas and was associated with numbness, mainly involving the entire right leg. There was some weakness and difficulty in controlling the movements of this leg. One possible episode of confusion occurred at night, but there was no delirium nor loss of consciousness. There was no headache nor apparent fever. A physician was not consulted. Numbness and weakness of the right leg persisted, but slight improvement was noted after a week and significant improvement after a month. P.G. was given a complete neurological examination by Dr. Wells on the same day as R.S. At this time, it was noted that he tended to throw his right leg outward in walking and that when he lay supine there was outward rotation of his right foot. The upper superficial abdominal reflexes were equivocal bilaterally, while the lower abdominals were both absent, as were the cremasterics. There was no evidence of wasting or weakness of either lower extremity, but resistance to passive stretch was slightly increased in the right. There was some diminution of sensation over the entire right lower extremity. Deep tendon reflexes were increased in the right leg and the Babinski and Chaddock signs were positive on the right. The electroencephalogram was interpreted as showing a pathological picture because of minor paroxysmal slow activity and because of spiking. The conclusion of the neurologist was that the above positive findings provided definite evidence of disease of the cerebral hemispheres, most probably involving the left.

LABORATORY STUDIES

On the afternoon of the 2nd day of the illness of R.S., a blood specimen was obtained. The virus of EEE was isolated from the serum of this specimen. The amount of virus present was insufficient to cause apparent illness or death in adult mice, but 4 of 25 1-day-old mice succumbed to infection 2 days after intracerebral inoculation and the virus was recovered from the brains of these mice. Identification was made by complement-fixation (CF) test, with standard immune mouse sera. An acetone-ether extracted antigen was prepared from suckling mouse brain infected with the virus from R.S. This was tested by CF, together with a similar antigen prepared from our prototype laboratory strain of EEE, against four standard sera, EEE, WEE, Semliki and West Nile. The results are shown in Table 1.

TABLE 1

Identification of R.S. virus by complement fixation

Antigen	Serum titer against 8 units of antigen			
	EEE serum	WEE serum	Semliki serum	West Nile serum
EEE prototype	512	4	0*	0
R.S. virus	512	8	0	0

* Less than 4.

Results expressed as reciprocal of serum dilution giving complete or almost complete fixation of 1.5 units of complement.

TABLE 2

Neutralization tests against EEE and Semliki Forest viruses

Serum	EEE		Semliki Forest
	Adult mice* Mice prot./ mice inoc.	Suckling mice† Mice prot./ mice inoc.	Suckling mice‡ Mice prot./ mice inoc.
R.S.—acute	0/6	0/8	8/16
R.S.—convalescent	5/6	8/8	10/16
P.G.—"convalescent"	5/6	8/8	0/8

* Virus dose 280 LD_{50}—mice inoculated by i.c. route.

† Virus dose 160 LD_{50}—mice inoculated by i.p. route.

‡ Virus dose 100 LD_{50}—mice inoculated by i.p. route.

TABLE 3

Hemagglutination-inhibition against various group A arbor virus antigens

Antigen	Serum titer*		
	R.S. acute	R.S. convalescent	P.G. "convalescent"
EEE	0†	160	160
WEE	0	0	0
VEE	0	0	0
Sindbis	0	0	0
Middelburg	0	0	0
Semliki	0	80	0
Chikungunya	0	20	0
Mayaro	0	0	0
AMM 2021	0	20	0
AMM 2354	0	40	0

* Reciprocal of serum dilution giving complete inhibition of 8 units of antigen.

† Negative at serum dilution of 1:20.

TABLE 4

Complement-fixation test against EEE antigen

Serum	CF titer*
R.S. acute	<4
R.S. convalescent	<4
P.G. "convalescent"	4

* Reciprocal of serum dilution giving complete or almost complete fixation with 8 units of antigen and 1.75 units of complement.

A convalescent bleeding was taken from R.S. 5 weeks after the onset of his illness, and a specimen was obtained from P.G. at the same time. No earlier specimen was available from P.G. Neutralization, hemagglutination-inhibition (HI) and CF test studies were carried out with these sera. The results are presented in Tables 2, 3 and 4. We are indebted to Dr. J. Casals for the detailed HI studies with a large number of antigens of Casals' group A of the arthropod-borne (arbor) viruses.[2, 3]

Neutralization tests were done against EEE virus in both adult and suckling mice and against Semliki Forest virus in suckling mice (Table 2). EEE and Semliki Forest viruses are both members of group A. In the EEE tests, the acute-phase serum from R.S. showed no protective antibodies while his convalescent specimen and the one from P.G. were both clearly protective. The significance of the partial protection shown

by both specimens from R.S. against Semliki virus will be discussed later.

In Table 3, the HI results against EEE antigen again show a clear-cut conversion for R.S. The single specimen from P.G. had the same titer (160) as did the convalescent serum from R.S. In this test, antigens from 10 viruses of the A group were used. It should be noted that in the case of P.G., the test was completely specific for EEE whereas the convalescent serum from R.S. reacted with several antigens, although the titer with EEE was the highest. The significance of this difference in response will also be discussed later.

The CF results in Table 4 are of interest since they indicate the weakness or inadequacy of this test under some circumstances. EEE virus infection would never have been suspected on the basis of CF response in the case of R.S., and a titer of 1:4 for P.G. would have been considered of dubious significance in the absence of other serologic data.

DISCUSSION

Human infections with EEE virus in the northeast United States have been thought to result usually in serious, if not fatal, central nervous system complications.[4] The two instances cited here indicate that this is not always true.

The infection of R.S. is well documented in all aspects except the source since he was exposed both in the laboratory and in the epidemic area. The clinical picture is of interest in that it consisted of a rather severe systemic infection without any overt manifestation of CNS involvement. The significance of the electroencephalogram findings cannot be assessed. The neutralization test and HI studies carried out with the two serum specimens from R.S. reveal a previous infection with another group A arbor virus (Tables 2 and 3). This can be said unequivocally to have been Semliki Forest virus, since R.S. had had laboratory contact with that virus over a period of several years and the neutralization test showed the presence of antibodies against the virus in both the acute-phase and convalescent sera. Although no HI antibodies to any group A virus were detected in the first bleeding, the convalescent specimen gave a significant titer against Semliki as well as lower titers against three other viruses of which two are rather closely related to Semliki. This pattern is quite at variance with that shown by P.G., where a completely specific EEE response occurred. HI studies carried out by Dr. J. Casals of these laboratories with several other human serum specimens obtained from the New Jersey area have also shown a completely specific EEE response. The development of detectable HI antibodies against Semliki by R.S. following EEE infection can be attributed to an anamnestic response to an immunologically related virus antigen.

Dr. Casals' studies have shown that a marked degree of protection against one group A virus can be conferred on experimental animals by preceding immunization with another, even distantly related, virus of the same immunologic group.[5] It is interesting to speculate on the possibility that in the case of R.S. the pre-existing immunity to Semliki Forest virus may have provided sufficient cross-immunity to modify the subsequent EEE infection and protect the central nervous system.

From the serologic findings, it is apparent that P.G. was infected at some time with EEE virus; but it is, of course, impossible to date the infection in the absence of either virus isolation or an acute-phase serum specimen. However, the fact that his serum HI titer was the same as the convalescent titer of R.S. suggests, though it does not prove, a relatively recent infection. The relationship between the acute episode experienced by P.G., with the resultant positive neurological findings, and his EEE infection will necessarily remain suggestive but conjectural.

One final point which deserves emphasis concerns the relative usefulness of the CF and HI tests. As stated above, the CF test was of no value in the diagnosis of these two cases. Although it might be argued that the low titer CF result shown by P.G. could be explained by assuming that his was an old rather than recent infection, no such contention can be made in the case of R.S. We feel that these results indicate that the test is an unreliable tool to use, not only for survey for recent inapparent infections, but even for the diagnosis of relatively mild, atypical infections with the virus of EEE. In contrast, the HI test, which has the same advantages of speed, simplicity and economy, not only seems much more sensitive than the CF, but proves to be highly specific in the absence of a preceding infection with a related virus.

SUMMARY

The clinical picture and laboratory findings in the case of a purely systemic infection with EEE virus are described. Another case is described in which the relationship between the EEE infection and the clinical picture is problematical.

Virus was isolated from the blood of the first case, and serologic studies were carried out with paired sera from that case and a single specimen from the second case. Specific serologic diagnosis was possible by means of either neutralization or HI tests, but the CF test was found to be too insensitive to detect significant antibody levels in these sera.

Previous infection with a related virus was recognized to have occurred in one case and to have influenced the pattern of HI response. The possible protective effect of this previous infection against serious consequences of the current EEE infection is mentioned.

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AN OUTBREAK OF INFLUENZA B IN PANAMA

JOHN E. CRAIGHEAD,* ALEXIS SHELOKOV, JOHN E. VOGEL AND PAULINE H. PERALTA

Middle America Research Unit, Balboa Heights, Canal Zone, and Laboratory of Tropical Virology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland

This report describes an outbreak of influenza type B which occurred on the Isthmus of Panama during June and July of 1959. Some factors possibly contributing to the appearance and spread of the disease in this outbreak are noted. In addition, tissue culture techniques feasible for the study of influenza by field laboratories are described.

MATERIALS AND METHODS

Virus isolation. Throat swabs from acutely ill patients were agitated in screw cap vials containing 4 ml of a diluent composed of 10% sterile skimmed milk in Hanks' balanced salt solution with antibiotics. Aliquots of these specimens in the amounts of 0.2 ml were introduced into each of two to four rhesus monkey kidney cell culture tubes. Usually specimens were inoculated into cell cultures shortly after arrival in the laboratory, although occasionally they were stored at about −55°C for brief periods before testing.

The cell cultures were received by air at weekly intervals.† On arrival they were washed with Hanks' solution and for their maintenance Eagle's basal medium with antibiotics, but without serum, was added. Tubes were incubated in stationary racks at 36 to 37°C and were observed daily for cytopathic effect (CPE).

Following inoculation, attempts to demonstrate hemadsorption[1] were made at 5- to 6-day intervals or at a time when definite CPE became evident. Two-tenths ml of Hanks' solution containing a mixture of 0.2% chicken and 0.2% guinea pig erythrocytes was added to each tube and hemadsorption was evaluated, according to criteria previously described,[2] after incubation for 15 to 20 minutes at room temperature. Fluids and cell scrapings from tubes exhibiting CPE or hemadsorption or both were passaged into fresh cell cultures.

Virus identification. Isolates were identified by either hemadsorption-inhibition (HAdI)[2] or hemagglutination-inhibition (HI) tests employing chicken or guinea pig erythrocytes and rooster antisera prepared against both A_2/Jap/305/57 and B/GL/54.‡ In the HAdI test cell cultures of the second passage of an isolate were utilized at a time when definite CPE was evident; hemadsorption was evaluated following incubation of the cultures for 15 minutes in the presence of 0.8 ml of a 1:20 dilution of specific antiserum. An isolate was identified if hemadsorption was completely inhibited by the antiserum and if hemadsorption occurred in similarly treated controls in the absence of the specific antiserum. The HI test was carried out by standard methods[3] utilizing culture fluids of the second or subsequent tissue culture passage of an isolate.

HI tests on patients' sera. Acute and convalescent sera were tested by HI with egg allantoic fluid antigens prepared against both the Panama prototype type B isolate strain and a strain of type A_2 influenza virus isolated from a specimen collected in British Guiana in June, 1959. Inactivated (56°C for 30 minutes) sera were adsorbed with a 10% suspension of kaolin and paired serum specimens were tested in parallel by the Salk pattern technique[3] using chicken erythrocytes and 4 to 8 hemagglutinating units of antigen. A fourfold or greater difference in HI antibody titer between the acute and convalescent specimens was accepted as evidence of infection.

BACKGROUND

The Canal Zone and the two major cities of the Republic of Panama, Panama City and Colon, are located roughly 9° north of the equator in the narrowest portion of the Isthmus of Panama. Panama City, with a population of over 250,000, is situated at the Pacific terminus

* Present address: Department of Pathology, Peter Bent Brigham Hospital, Boston 15, Massachusetts.

† From Microbiological Associates, Inc., Bethesda, Maryland.

‡ Influenza Diagnostic Kit supplied by Communicable Disease Center, U. S. Public Health Service, Chamblee, Georgia.

of the Canal, while Colon, a city of approximately 55,000, lies adjacent to the Canal in lowlands along the Caribbean coast. About 46,000 persons live within the Canal Zone—about 31,000 United States citizens and 15,000 natives of the Isthmus. The major population centers of the Canal Zone are located in proximity to Panama City and Colon and are separated by some 50 miles.

Throughout the year the climate of the Isthmus is warm and humid. There are, however, distinct wet and dry seasons which are locally termed "winter" and "summer" respectively. From January through April little rain falls and the Isthmus is bathed by warm tradewinds from the northeast. During the wet season between May and December heavy rainstorms occur almost daily. Table 1 gives weather data for the Pacific coastal area for the first 8 months of 1959 as recorded at Balboa Heights, Canal Zone.

TABLE 1

Monthly weather data, Pacific coast of Panama, January–August, 1959*

Month	Rainfall (total hours)	Precipitation (total inches)	Temperature (average °F)	Relative humidity (average %)
January	4	1.1	79.2	75.5
February	0	0	79.9	71.7
March	0	0	81.5	69.7
April	7	1.5	82.4	72.4
May	40	9.2	80.0	82.6
June	48	4.6	78.4	87.3
July	39	5.6	79.4	86.8
August	52	7.6	78.7	86.3

* Recorded at Balboa Heights, Canal Zone.

THE OUTBREAK

In early June, 1959, a sharp increase in the occurrence of influenza-like illness was noted by physicians in the two major cities of the Republic of Panama and in the Canal Zone. The disease was first recognized soon after the onset of the "wet" season (Table 1) and about 1 month following the opening of the Republic of Panama schools and the Canal Zone "Latin American" schools.*

Clinical influenza was first reported from the Caribbean coastal city of Colon on June 10th and from the Pacific coast area a few days later. During the latter part of June and early July the disease was widespread on the Isthmus. Specimens collected during this period yielded only type B influenza viruses, thus confirming the clinical impression.

Following the outbreak, Canal Zone school records were inspected since it was thought that information on student absenteeism would give an indication of the rapidity and extent of disease spread. A sharp rise in student absences was recorded during June and the first weeks of July. Peaks in absenteeism in schools situated near the two coasts were separated by about 2 weeks as shown in Figure 1. Since virus isolations from both areas coincided with these peaks it is reasonable to assume that influenza was to a large extent responsible. School records did not indicate a similar rise in absences during the comparable period of 1958. These observations substantiate the impression that the outbreak spread from the Caribbean to the Pacific side of the Isthmus.

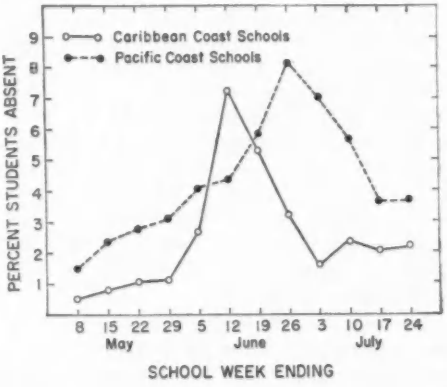

FIG. 1. Percentage of students absent from Canal Zone schools during May, June and July, 1959.

The clinical illness was characteristic of the influenza syndrome and the symptomatology did not appreciably differ from that occurring in temperate climates. Although illness occurred in all age groups, the incidence appeared to be highest in younger children since absenteeism during June in elementary schools was sub-

* The schools in the Republic of Panama and the "Latin American" schools for non-United States citizens residing in the Canal Zone are in session from May through January. The Canal Zone schools for United States citizens are on the North American schedule and were recessed at the time of the outbreak.

stantially greater than in high schools. No influenza-associated deaths were reported in the Canal Zone during 1959.

LABORATORY STUDIES

Virus isolation and identification. Twenty-five viruses, antigenically similar to B/GL/54, were recovered from the total of 59 specimens tested. Rapid isolation was facilitated by tissue culture techniques since virus-positive cultures usually exhibited characteristic CPE on the 3rd to the 5th day following inoculation. Inasmuch as attempts to demonstrate hemadsorption were carried out on the 5th or 6th day, in only one instance was a culture found to hemadsorb before the appearance of CPE. Hemadsorption allowed, however, for the preliminary identification of cytopathogenic agents since its presence was a good indication that the isolate was a myxovirus and presumably influenza.

Fifteen of the 25 isolates were identified by the HAdI test, while the remainder were identified by HI. In a few instances both tests were employed. Although HI was used for most of the early isolates, the HAdI test was later found to be preferable since it was less time consuming.

Hemagglutination by the Panama prototype isolate strain and by the B/GL/54 antigen was found to be almost equally inhibited by antiserum prepared against the local strain, the B/GL/54 virus, and a type B strain isolated in Maryland during 1959.* The B/GL/54 antiserum was utilized routinely for purposes of identification.

HI tests on patients' sera. Fourfold or greater rises in HI antibody titers were demonstrated in 27 of the 39 pairs of acute and convalescent sera tested. Twelve of the 13 persons, from whom virus isolations were made and from whom paired serum specimens were available, developed significant rises in HI antibodies. Serological evidence of infection was found in 15 additional persons from whom no virus was isolated. No HI antibody rises to the type A_2 antigen were demonstrated.

DISCUSSION

Beginning in late 1958 and during 1959 outbreaks of influenza due to types A_2 and B influenza viruses were reported from many parts of the world.[4] The A_2 viruses recovered have thus far been found to be antigenically similar to the Asian strain pandemic in 1957–58 and their appearance was fully anticipated. The widespread occurrence of influenza due to type B strains was consistent with the established periodicity of this virus type.

The etiology of several other influenza outbreaks in the American tropics during 1959 was determined. Limited studies carried out in this laboratory on specimens collected in Costa Rica and British Guiana demonstrated influenza virus activity in these countries. Type A_2 virus strains were isolated and serological evidence of infection with these, but not with type B viruses, was found in specimens collected in Georgetown, British Guiana, during a severe epidemic occurring in May and June. Serological studies of paired serum specimens collected in the course of a live poliovirus vaccine surveillance program in San Jose, Costa Rica, indicated that both types A_2 and B influenza viruses were active in that city during May, June, and July.[5] Spence[6] reports isolation of type A_2 virus strains in July and August in Trinidad. Outbreaks of clinical influenza, usually not confirmed by laboratory studies, were reported by national authorities in several other countries in the American tropics during 1959.

Many of the epidemiological factors contributing to the dissemination and spread of influenza viruses remain poorly understood. Factors considered of importance in temperate climates may differ from those operating in the tropics. While the time of introduction of the virus responsible for the Panama epidemic is not known, it is possible that the opening of schools and the seasonal changes which preceded the outbreak contributed to the spread of the disease. It is worthy of note that the British Guiana epidemic likewise became evident shortly after the onset of the wet season.

In this study tissue culture proved to be a technically simple, practical, and rapid system for virus isolation. Takemoto *et al.*[7] and Mogabgab *et al.*[8] have demonstrated the usefulness of monkey kidney tissue culture for the isolation of type B influenza viruses. Our results further substantiate the findings of these workers. Since the characteristic cytopathic effect of influenza type B viruses became evident soon after inocu-

* Supplied through the courtesy of Dr. R. M. Chanock, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland.

lation, hemadsorption did not assist in detecting viral growth in cell cultures. The technique of hemadsorption-inhibition facilitated the rapid serological identification of cytopathogenic agents.

Classical isolation techniques for influenza viruses are not feasible for many laboratories in tropical areas. A major problem is the unavailability of sufficient numbers of embryonated eggs of the proper age. In addition, preparation of specific standardized antisera is impractical for the small laboratory occasionally called upon to study an outbreak of influenza. The methods described here, utilizing air-transported, commercially-produced tissue culture tubes and diagnostic reagents available from the Communicable Disease Center, U. S. Public Health Service, illustrate techniques applicable for the study of influenza outbreaks by laboratories with limited personnel and facilities.

SUMMARY

An outbreak of influenza occurring on the Isthmus of Panama in 1959 is described. Type B influenza viruses, antigenically similar to B/GL/54, were isolated and serological evidence of infection was demonstrated in a high proportion of persons tested. The outbreak began shortly after the opening of schools and the onset of the wet season and it is possible that these factors contributed to the spread of the virus. Tissue culture techniques were found to be practical for the study of influenza in this outbreak.

ACKNOWLEDGMENTS

The clinical observations and specimens from the Canal Zone patients were kindly made available to us through the co-operation of Drs. C. F. Abildgaard, A. M. Blanshaft, and D. E. Ford of the Coco Solo and Gorgas Hospitals. We wish to thank Drs. B. B. G. Nehaul and C. C. Nicholsen and Mr. M. A. Hussain for their assistance in the collection of specimens in British Guiana.

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ANTIVENIN AND ANTITOXIN IN THE TREATMENT OF EXPERIMENTAL RATTLESNAKE VENOM INTOXICATION (*CROTALUS ADAMANTEUS*)*

FRANK J. FISCHER, HOWARD W. RAMSEY, JOSEPH SIMON AND JOSEPH F. GENNARO, JR.

Cancer Research Laboratory and Department of Anatomy, College of Medicine, University of Florida

As recently as 1954 the antivenin used in this country for the treatment of snake bite and made from eleven snake venoms was found to offer negligible protection against intoxication by any of the venom antigens from which it was produced.[1] At that time a new antivenom preparation was developed by Wyeth Laboratories and was shown by in vitro tests (the hyperimmune antiserum and measured quantities of the venom were allowed to complex in vitro and the ability of the immune serum to inactivate the venom poison was shown by mouse survival after injection of this complex, the neutralized toxin) to be markedly superior to the older preparation.[2] This new antivenin has never been evaluated for its protection by the injection of a quantity of antivenin prior to the administration of a known lethal dose of venom, as was the older preparation. It is the first purpose of this investigation to demonstrate the degree of protection afforded by the new antivenin in this manner against the venom of the Eastern Diamondback rattlesnake of which 25% of the bites treated by the old antivenin resulted in death.[3]

Further, and more important, the efficiency of a hyperimmune antitoxin in modifying the local necrotic effect of the snake bite lesion has never been made clear. As a matter of fact, the school of therapy advocating hypothermia seems to have been developed in the general understanding that antivenin is not effective in combating the local necrosis which follows snake bite. This local necrosis is so marked that many people who have seen it insist that it is almost invariably due to the presence of clostridial organisms which have been claimed to exist in the mouth of the snake. For this reason several authors have also advocated the use of tetanus and gas gangrene antitoxins in the treatment of snake bites.[4-6] Because the mouth of the snake seems to provide an appropriate environment for such microbes, some individuals regard the administration of these antitoxins as more important than the use of antivenin itself. A study of the literature has failed to disclose any investigation into the protection offered by these antitoxins. Because of this an attempt has also been made here to evaluate the effect of these antitoxins in the modification of the local necrotic lesion and on survival in the experimental snake bite.

* This investigation has been made with the assistance of a grant from the Committee on Research, Council on Pharmacy and Chemistry, American Medical Association, and also one from NIH RG 7024.

METHODS

The venom used in these studies was milked from 10 snakes, frozen immediately and then dried under vacuum. Toxicity was established by intraperitoneal injection into 100 male Swiss mice averaging 20 to 22 g in weight. Groups of 15 to 20 mice were injected with 1, 2, 3, 4 or 5 μg/g mouse. Food and water were supplied ad libitum and any death occurring within 72 hours after injection was considered to be due to the venom. By this means, it was determined that the complete lethal dose,[7] *i.e.*, the lowest dose lethal to all members of the group (LD_{100}), was 4 μg/g mouse.

Protection tests. For tests of protection by new polyvalent antivenin (Wyeth), tetanus antitoxin (Wyeth), and polyvalent gas gangrene antitoxin (Lederle) against the lethal effect of venom, 212 male Swiss mice from 20 to 25 g in weight were used. In all cases 0.1 ml of the material to be assayed for its protective effect was administered intraperitoneally. Controls consisted of similar groups of animals into which physiological saline or normal horse serum was administered in the same manner. After 1 hour all animals were given a 3 LD_{100} dose of *Crotalus adamanteus* venom in saline solution injected intraperitoneally.

A separate series of experiments was performed to determine whether there was any intrinsic toxicity in the antivenin, the antitoxins, or in normal horse serum. Four groups of ten 20-mg male Swiss mice were injected with varying

amounts of proteins intraperitoneally with a dose estimated on a body-weight basis to compare with that given to a 70-kg human receiving five 10-ml doses of antivenin, an average dose for a severe snake bite.[8]

For tests of protection by new polyvalent antivenin (Wyeth), polyvalent gas gangrene antitoxin (Lederle), and tetanus antitoxin (Wyeth) in preventing local reaction, 12 white male New Zealand rabbits weighing approximately 5 lbs. were used. The backs of the rabbits were shaved and cleaned with alcohol. The shaven areas were divided bilaterally and intradermal injections of 0.25 mg, 0.5 mg, and 1.0 mg of venom were made on one side. After 24 hours the skin reaction was noted and photographed and 5 ml of the serum to be tested was injected into an ear vein. After 1 hour more, a second series of venom injections duplicating the first was made on the opposite side of the shaven back. Twenty-four hours after the latter injection these sites were also observed and photographed.

Microflora of the mouth and venom of reptiles. Cultures were obtained from the mouths and venoms of 24 Eastern Diamondback rattlesnakes (*Crotalus adamanteus*), 21 cottonmouth moccasins (*Ancistrodon piscivorus*), 2 coral snakes (*Micrurus fulvius*), 1 Gila monster (*Heloderma suspectum*), and from the mouths of 2 yellow rat snakes (*Elaphe obsoleta quadrivittata*), 1 black snake (*Coluber constrictor*), 1 boa constrictor (family Boidae), 1 anaconda (*Eunectes sp.*), 1 hognose snake (*Heterodon sp.*), 2 natrix (*Natrix sp.*), and 4 alligators (*Alligator mississipiensis*). Oral flora were sampled with swabs moistened with sterile 1% peptone broth. Venom samples were obtained in sterile 250-ml beakers (approximately 0.5 ml of venom) and were diluted with 5 ml of sterile 1% peptone broth and subsequently poured from the beaker into stoppered sterile vials. These were iced until cultured about 1 hour later. Care was exercised to see that the venom-peptone mixture was poured out of the opposite side of the beaker from that on which the venom was collected so as to minimize the possibility of contamination of the venom by the mouth of the snake. This method of collection is largely as has been described in the literature.[9, 10] Cultures were obtained from snakes recently collected (within 1 week) as well as from those which had been in captivity for some time. In the latter group many mouths were suppurative, a condition quite common in snakes handled and milked in captivity.[10]

A loop of the broth containing the mouth swabbings and a loop of the diluted venom were cultured on blood agar base slants, incubated 48 hours at 34°C and examined for growth. Additional aliquots of each sample were heated to 75 to 85°C for 20 minutes and inoculated into recently boiled Brewer thioglycollate broth, polymyxin-thioglycollate-sorbic acid broth,[11] and stab cultures in media composed of brain-liver-heart agar (2%), trypticase-soy agar (2%), and trypticase-sodium azide-chloral agar (2%). These five cultures were incubated at 34°C for 2 days and examined. A subsequent incubation period of 2 additional days was followed by a second examination.

The original 1% peptone broth cultures containing the mouth swabbings and the venom, respectively, were incubated at room temperature (25°C) for 96 hours and subcultured on blood agar base plates, incubated for 48 hours longer (34°C) and examined.

RESULTS

Protection against the lethal effect of the venom. All mice injected with rattlesnake venom died within 2.5 hours except those protected 1 hour prior to venom administration by an injection of antivenin (Figure 1). Only four animals of the 25 so protected died within 72 hours. The animals to which gas gangrene antitoxin and tetanus antitoxin had been given died as rapidly as those which had only received normal saline or horse serum. In these groups most deaths occurred within 2 hours and all animals were dead by 2.5 hours after the injection of the venom.

Protection against local necrosis. The marked skin response to the Eastern Diamondback venom injected intradermally (pronounced intradermal hemolysis, cytolysis, and ulcerative necrosis) was almost completely suppressed by the administration of antivenin (Figure 2). The animals which received tetanus antitoxin or gas gangrene antitoxin manifested a skin reaction to the venom equally as intense after the administration of these sera as before. In neither case, nor at any of the venom dosages, did there seem to be the least inhibition of the skin reaction. These lesions ranged in diameter from 1 to 3 cm, and those which resulted from the higher dosage always exhibited a loss of surface substance with a

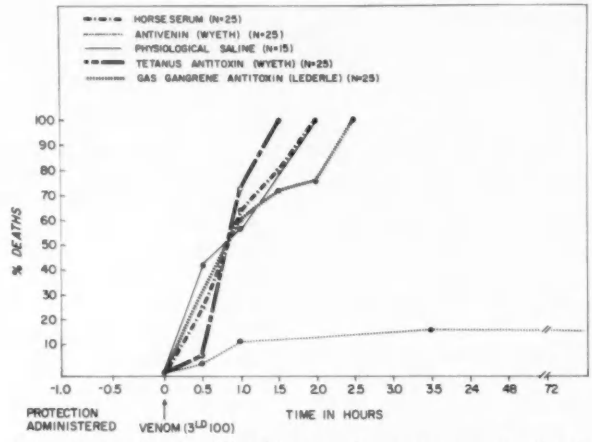

FIG. 1. Effect of polyvalent antivenin (Wyeth), polyvalent gas gangrene antitoxin (Lederle), tetanus antitoxin (Wyeth), normal horse serum, and physiological saline on the toxicity of *Crotalus adamanteus* venom (♂ 20-g mice).

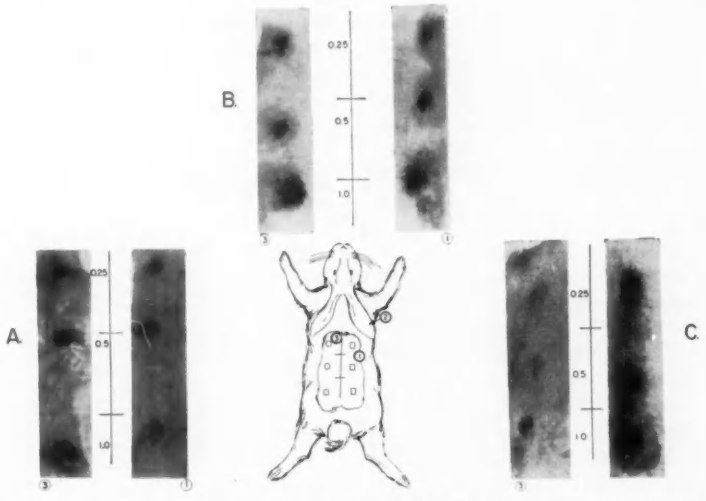

FIG. 2. Effect of polyvalent gas gangrene antitoxin (Lederle) *A*, tetanus antitoxin (Wyeth) *B*, and polyvalent antivenin (Wyeth) *C* on the local necrosis produced by *Crotalus adamanteus* venom.

moist appearance at the site and a sloughing of the deeper cutaneous tissues.

Microflora in the mouth and venom. Good growth was obtained from the cultures made from the mouths of all the reptiles tested except those most recently collected. The dominant flora were aerobic spore formers, micrococci, *Pseudomonas sp.*, coliforms, and fungi. Unlike cultures from the oropharynx, aerobic growths were rare in venom samples cultured. Anaerobic growth was not obtained in either case or in any instance by these methods.

DISCUSSION

It seems obvious from these results that no benefit is obtained in the treatment of rattlesnake bite with tetanus or gas gangrene antitoxin

against either the lethal or the local necrotizing effects of the venom. Only hyperimmune serum prepared for this purpose is of value, and its protection is marked in both respects.

The use of tetanus and gas gangrene antitoxins seems to be advocated on the grounds that "because of the natural environment and feeding habits of poisonous snakes, their mouths are always heavily contaminated with bacteria; it is inevitable that venom from these creatures will be infected."[2] Though this appears to be a sound assumption, investigators who have sampled the microfloral populations of the mouth of snakes in the wild have affirmed that these populations are remarkably small.[10] This conforms with the results obtained by Parrish *et al.*[9] and those of the present study. The latter have shown, in addition, that the organisms which do develop in the snake's mouth shortly after captivity and which rapidly increase in quantity in carelessly handled animals to such an extent that it may be impossible for the mouth to close do not include those of the anaerobic type. Moreover, consideration of the venom apparatus makes it clear that the poison secretion is not usually situated in the mouth of the snake but is conducted through it into the object bitten. Fresh venom, like most body fluids, is free of microorganisms. This can be shown if the venom is carefully collected for culture by "milking" into sterile containers directly or into sterile containers covered with a rubber dam under conditions similar to the actual snake bite situation. While it is true that clostridial organisms have been demonstrated in dried venom preparations,[12] the hypothesis suggests itself that contamination occurred during the preparation of the dried venom. This is to some extent substantiated by the investigation of Williams *et al.*,[10] whose study showed that dried venom contamination increases as preparation procedures are prolonged. In view of the precaution taken in the preparation of the venom used in this work and as a result of its essentially sterile character, it is not surprising that the necrosis which was demonstrated after its injection in the rabbit was not amenable to antitoxin therapy with clostridial antitoxins. The necrosis demonstrated here is, in fact, due to the cytolytic action of the native snake protein. In this respect this experimental situation is essentially similar to the actual snake bite in which, barring subsequent contamination, the necrosis is also due only to the action of specific cellular lysins which are immunologically unlike the microbial ones.

It seems clear that the most effective contemporary treatment for both the systemic and local sequelae of snake bite is antivenin. Though the use of this preparation carries with it the dangers attendant on the injection of large quantities of foreign protein, these are largely minimized in view of the disease produced by a serious bite. In any event, it does not seem advisable to increase the foreign protein burden by the administration of large quantities of additional protein on an *a priori* basis without *specific evidence* of its need.*

SUMMARY

The protection afforded by the new polyvalent antivenin preparation (Wyeth), tetanus antitoxin (Wyeth), and polyvalent gas gangrene antitoxin (Lederle) against death in the mouse and local necrosis in the rabbit produced by the venom of the Eastern Diamondback rattlesnake (*Crotalus adamanteus*) was investigated. The microbiological populations of the mouth and venom of several poisonous and non-poisonous North and South American reptiles were sampled and specifically cultured for spore-forming anaerobes.

While the new polyvalent antivenin gave marked protection against death and local necrosis produced by rattlesnake venom, the polyvalent gas gangrene antitoxin and tetanus antitoxin offered no protection against either. Aerobic growth appeared to a greater extent in the oropharynx than in the venom. Anaerobic growth was not obtained in either case.

These results, especially in regard to the relatively sterile nature of the snake venom, suggest that addition to the foreign protein burden in the treatment of snake bite by the administration of large quantities, *i.e.*, therapeutic dose, of tetanus or gas gangrene antitoxin is unnecessary and indeed contraindicated unless *laboratory evidence* of clostridial contamination exists. This does not

* Prophylactic doses of tetanus and gas gangrene antisera advocated by the manufacturers are 1,500 units (~ 1 ml) of the former and 21,500 units (~ 20 ml) of the latter. Treatment dosages for the first 24 hours are given as a minimum of 250,000 units (50–60 ml) of the former (200,000 units given intravenously) and 18 to 44 doses (200–500 ml) of the latter, administered intravenously and intramuscularly.

imply the prompt prophylactic use of antitetanus serum or tetanus toxoid should be abandoned.

ACKNOWLEDGMENTS

The authors are indebted to Lamar Batts and William Gleason of the Ross Allen Reptile Institute, Silver Springs, Florida, for their assistance in handling the reptiles used in this study. Also gratefully acknowledged is the help given by the Department of Medical Illustration and by Robert Morris, Hollis Hall, Doris Gennaro, and Bobbie Bagley.

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STUDIES ON KURU

I. The Ethnologic Setting of Kuru

D. CARLETON GAJDUSEK and VINCENT ZIGAS

National Institute of Neurological Diseases and Blindness, National Institutes of Health, Bethesda, Maryland, and Public Health Department, Territory of Papua and New Guinea

Kuru is a recently recognized disease entity which is completely restricted to Melanesian natives of one small sub-stock in the Eastern Highlands of Australian New Guinea.[1-22] The disease presents a problem, almost unique in medicine, of an entire race of people afflicted with a rapidly progressive, acute degenerative disorder of the central nervous system, apparently of genetic origin, which accounts directly for about one-third of all deaths in the community and both directly and indirectly for almost one-half of all mortality. This ethnographically and geographically localized illness occurs among natives of the Fore linguistic and cultural group, or their immediate tribal neighbors who intermarry with them, and is presumably the result of a single mutation whose selective advantages have not, as yet, been elucidated.

In the Fore language, "kuru" is a word which is also used to designate the trembling associated with fear or cold. It is, primarily, a disease of motor coordination with ataxia somewhat akin to the trunkal ataxia of midline cerebellar lesions. Excitement of any sort causes exaggeration of involuntary movements which disappear in sleeping. The onset is insidious, without antecedent febrile illness, the patient himself being aware of his clumsiness in walking before members of his family notice it. The subsequent course of the disease can conveniently be divided into three clinical stages. In the first stage, while the patient is still ambulatory, there is a fine irregular tremor akin to shivering, accompanied by a progressive locomotor ataxia, occasional myoclonic jerks and emotional lability which is predominantly euphoric. In the second stage the patient requires a stick for locomotion and later can only walk when supported by others. Tremors are still coarse and irregular, and choreiform and athetoid movements and myoclonic shock-like jerks are present. Dysarthria appears, and emotionalism, with easily provoked inordinate laughter, persists. Strabismus develops often accompanied by nystagmoid eye jerks. Tendon jerks are usually hyperactive and there may be unsustained ankle clonus. These two ambulatory stages pass to a third stage when the patient is sedentary, unable to walk even with support, and is dragged from the low kunai grass house to sit out of doors. When he loses balance even when sitting, he is usually left to lie inside until death ensues. Muscular fibrillations appear in this terminal phase. Muscle tone may be normal or may alternate between rigidity and flaccidity. The patient is aphonic and dysphagia leads to starvation. Decubitus ulcers develop and terminal bronchopneumonia is frequent.

Numerous attempts at therapy have been without success and with the exception of rare remitting and exacerbating cases and extremely rare apparent recoveries most patients die within 6 to 12 months after the onset of the disease.

All cases of kuru have occurred within the boundaries of the region defined in the map in Figure 1, with a few very significant exceptions. Those include cases that have developed near both the Papuan and New Guinea coasts, far north in the Kamano area near Kainantu, and at the Lufa patrol post. All of these exceptional cases, however, have been in kuru region natives who have been living and working away from home for various lengths of time. With large numbers of potential kuru victims—*i.e.*, natives from the kuru region who presumably carry the kuru gene—now residing outside the kuru region, more cases may be expected to appear in other parts of New Guinea.

We have encountered three cases of a somewhat kuru-like neurological disorder with progressive, severe ataxia, involuntary movements and disordered coordination in the Wabag-Laiagam region of the Western Highlands of Australian New Guinea, some 170 air miles from the kuru region.[18] Patients with this new Western disease are currently being studied and

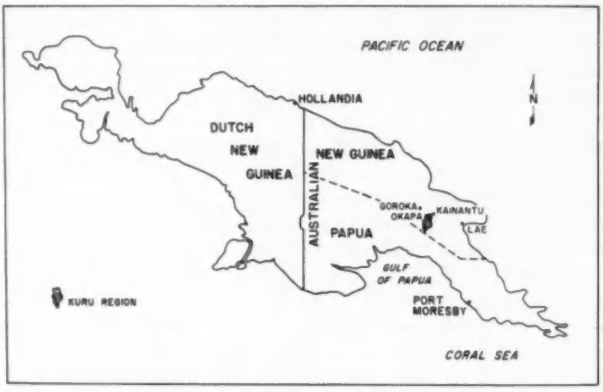

FIG. 1. The kuru region in Australian New Guinea

the epidemiology of the disorder is under investigation. No neuropathological observations have yet been possible. Other kuru-like tremor cases have recently been seen in the Mendi region of the Southern Highlands of Australian New Guinea.

Within the kuru region, kuru causes the death of about 1% of the population annually. The disease principally affects mature females, and adult males are rarely stricken. About one-fourth of the cases occur in children of both sexes. In some small hamlets the annual death rate attributable to kuru has reached 5% to 10%; here the female to male ratio may be as high as three to one. The characteristic distribution of cases in relation to age and sex strongly suggests that some undetected ethno-environmental variables are operating and that endocrine factors play an important role in the pathogenesis.

In the study of few diseases are complex geographic, ethnologic and demographic factors so pertinent to the medical investigator. Kuru's massive inroads upon the population and its rapidly progressive course to death must bring it to the forefront of interest and concern among neurologists, epidemiologists and geneticists. Although its etiology remains undetermined, we have been forced to conclude the likelihood of genetic predisposition from very early in our studies[1] and to admit the possibility of rather a simple single gene mechanism;[4] yet, there still remain unexcluded toxic, deficiency, and infectious etiological possibilities.

Detailed studies of human genetics in the kuru region are still in progress; however, a single case of kuru in a Melanesian from a region unaffected by kuru or in a Caucasian living with the Fore people and exposed to the kuru environment would demolish the genetic hypothesis. We have observed no such case as yet. Even if genetic constitution is primarily responsible for the disease, there are rather certainly environmental variables which affect the expression of the disease and contribute to the mechanism of its pathogenesis.

Remarkable in the kuru situation is the high frequency which a lethal gene has attained in a whole race of people. To have reached such frequency the heterozygous carriers of the gene must possess a marked adaptive advantage. These carriers of the hypothetical gene for kuru are not yet identifiable by any biochemical test, and any member of the kuru region population must therefore be regarded as a possible carrier. In consequence of this, the government of Australia has declared a "genetic quarantine" of the kuru region. This eugenic policy, pragmatically unenforceable, is without precedent anywhere.

The epidemiological, clinical, pathological and laboratory findings in kuru have been summarized in earlier communications.[1-19] In the current series of papers we shall present more extensive field and laboratory investigations on kuru. This introductory paper includes further exposition of the complex ethno-environmental situation surrounding kuru, a report of the method of case reporting being used, an interim summary of the current incidence and statistics of kuru, and a presentation of previously unrecorded ethnographic data pertaining to the past history and origin of kuru.

COMPREHENSIVE RECORD OF ALL CASES OF KURU

The serial documentation of every kuru case which occurs in the kuru region was begun in March, 1957. A vigorous attempt at documenting cases back to January 1, 1957, was made. Such cases have been termed "observed" kuru cases. Records of earlier cases are obtained by anamnestic technique and an attempt has been made to exhaust the tribal memory of kuru cases in interviews in every hamlet of the kuru region. These are directed towards exhausting the recall for kuru deaths of all potential informants. When this record of deaths from kuru prior to January 1, 1957, is complete, the combined "observed" cases and those documented from memory as having resulted in death before 1957 should constitute the "complete" record of kuru.

Kuru deaths prior to 1957. The record of cases which terminated fatally before 1957 is now being compiled and our already existing field notes comprise, we believe, the greater part of all recalled kuru cases, records of which shall ever be available to us. All kuru hamlets have been surveyed, fully half of them with great intensity. We estimate that we already have documented well over half of all kuru cases that can be elicited from the memories of all individuals of the kuru region by exhaustive interrogation.

The cases terminating fatally before 1957 include all those verified by cross-checking information with at least two independent informants. Fortunately, the natives recognize the unique clinical syndrome of kuru and there is very little inconsistency in their reporting of past deaths from kuru. Those few cases which remain unverified, for which conflicting accounts have been obtained, are placed in a separate file and not entered with the record. A second section of this record lists all individuals with kuru who are said to have had active disease before 1957 and have recovered from their illness. A great majority of such persons can be found alive and well today, and all such individuals have been examined and interviewed.

Clinical and epidemiological data on those dying prior to 1957 are as extensive as the circumstances of their collection permit them to be. Thus, the patient's genealogy is obtained, and all data possible to obtain by interview about the time sequence and the course of the patient's illness are recorded. Place of onset, place of residence during the illness, and place of death are recorded with the specific ground name of the patient's dwelling and food gardens in each case. Peculiarities in the patient's clinical course and any unusual facts recalled about the patient are included. Age is estimated and the case is dated as accurately as our chronology in the kuru region permits.

Kuru deaths since January 1, 1957. Patients with kuru dying since January 1, 1957, are separated into three categories:

1. *S cases* include kuru subjects who have been brought to the Okapa or Kainantu Kuru Research Centers for extensive study. These cases have full clinical charts and are equivalent to hospital "in-patients."

2. *E cases* include kuru subjects seen and examined by a member of the Kuru Research team in their local villages. In many cases they have been seen repeatedly and blood specimens and other specimens have been collected. These patients correspond to hospital "out-patients."

3. *N cases* include those reported to us—the reports of which we have been able to verify by repeated cross-checking. However, they have not yet been seen and examined by a member of the Kuru Research team. In many instances, reliable native assistants have visited these patients repeatedly and reported to us the progress of their disease.

At the present time the "observed" case record is complete for the years 1957 through 1960 and is being continued.

Recovered patients who have had active disease since January 1, 1957, are included in the file of recent cases. This group, obviously, contains some misdiagnoses, some cases of hysterical mimicry of the kuru syndrome, and instances of remission of true kuru; but at the current state of our knowledge we are unable to separate these three categories.

On the charts of S and E cases of kuru occurring since January 1, 1957, many additional data are available to those recorded for patients dying before 1957. These include detailed anamneses, physical examinations, laboratory procedures and full clinical charts, along with all pertinent ethnological and epidemiological observations which were made. Information on the charts of the N cases is usually similar to that obtained for patients dying before 1957, with the added data

TABLE 1

Age and sex summary and distribution of 804 studied cases of kuru

Type of case*	4 years		5–9 years		10–14 years		15–19 years		20–29 years		30–39 years		40–49 years		50 years		Total all ages			Current status	
	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	Both sexes	Alive	Dead
S	0	2	14	9	12	13	1	6	25	5	28	2	17	0	5	0	102	37	139	16	123
E	2	0	28	26	18	15	14	5	72	6	87	1	32	0	21	0	274	53	327	146	181
N	1	0	43	16	24	16	12	9	70	6	85	0	35	0	21	0	291	47	338	54	284
Total	3	2	85	51	54	44	27	20	167	17	200	3	84	0	47	0	667	137		216	588
Both sexes	5		136		98		47		184		203		84		47		804		804		
F:M ratio	1:0.67		1:0.60		1:0.81		1:0.74		1:0.10		1:0.015		0		0		1:0.205				

* "S" refers to individuals studied in research centers; "E," to those examined in the villages by members of the team; and "N" includes cases reported upon by reliable native assistants.

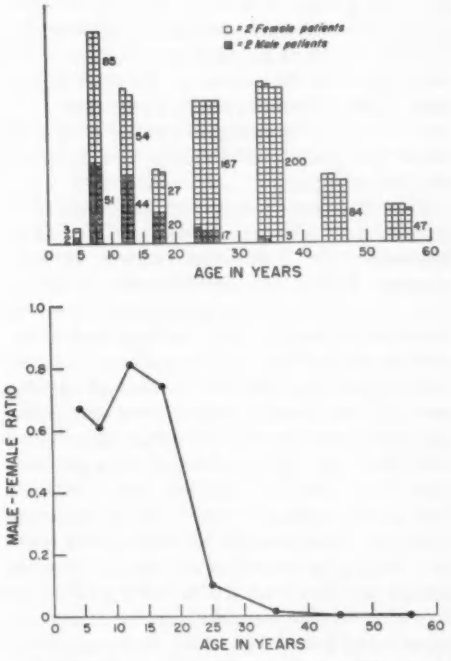

FIG. 2. Age and sex distribution of 804 studied cases of kuru.

on clinical course and with associated facts which the contemporaneous occurrence of these cases made it possible to obtain.

A summary of the S, E and N observed kuru cases for 1957 through 1960 is tabulated in Table 1. The age and sex incidence data, as well as the female to male ratio for all cases (S, E and N combined), "observed" since January 1, 1957, are presented in Figure 2.

OBSERVATIONS ON THE PAST HISTORY OF KURU

A crucial question posed by all observers of the kuru situation is, "How long has kuru been occurring among these people?" We have stated elsewhere that the disease has occurred throughout the Fore region for the "whole of tribal memory." It is obvious that this statement needs further clarification.

On first inquiry as to the occurrence of kuru in their villages, the natives of almost all of the Fore hamlets have stated that it has been present for a "long time"; but this they soon modify to mean that in recent years it has been becoming an increasingly severe problem and that in the early youth of our oldest informants there was no kuru at all. The critical examination of such reports requires a preliminary account of (1) our interviewing technique, (2) our choice of informants, (3) the estimated reliability of our informants, and (4) the time scale among the natives. Additional speculation as to the length of time kuru has been present demands an analysis of the significance of myths and legends and complex sorcery and counter-sorcery systems as sources for rough estimation of elapsed time and a critical evaluation of information from other sources from which the past history of kuru may be evaluated.

Interviewing, choice of informants and estimated reliability. In every community inhabited by the Fore we have obtained the history of kuru deaths

in the past as well as in the present. By persistent inquiry of successively older informants we have always been able to find reports of individuals whose death occurred an estimated 25 to 35 years ago. Such reports can be confirmed by cross-checking independently with other informants. Often reports of deaths in the distant past can be dated at least 35 years ago, but beyond this estimated remoteness in time we have obtained no reports of recalled kuru deaths. The absence of memory for earlier kuru in these communities and the diminishing number of cases reported for periods before the last decade would lead to the conclusion that kuru was only a recent occurrence among these people. However, we are forced to draw quite the contrary conclusion. This apparent paradox is explained by the following observations: (1) the regularity of the reports of cases for 25 and even for 35 years ago in widely scattered communities, (2) the approximate nonvariant incidence of the disease from year to year in the recent past years over which memory for all deaths is well preserved, (3) the apparent diminution in past incidence of kuru as first noted only in anamnestic data for the more remote past where tribal memory has begun to fail and sufficiently aged informants are difficult to find, and, finally, (4) the crucial observation that accurate memory for relative order of occurrence of deaths and for the causes, as assigned in the natives' nosological classifications, is a characteristic of late adolescence and adulthood not found in childhood or early adolescence. For the immediate past which has been under our own direct observation we find the recall of older children and even young adolescents often faulty, inconsistent and inaccurate, whereas that of older adolescents and adults is detailed, consistent and accurate. The errors of memory of our informants under about 15 years of age are remarkable when contrasted to the reliability of information from slightly older informants. As an indication that this is not a new phenomenon we note a similar unreliability of information pertaining to a period over 10 years ago from 25-year-old informants whose recall over the past decade is proven reliable by independent cross-checking. We may, therefore, conclude that before marriageable age children and youths pay little heed to assigned causes and other details of deaths in their community, whereas, as they approach the age of increased participation in the economic, political and reproductive life of their communities, they note these facts with new sustained interest.

From such a conclusion we would expect reliable historical information about kuru to extend back some 5 years for 20-year-old informants, 15 years for 30-year-old informants and 25 or 35 years for informants 40 or 50 years old respectively. Very few informants over 50 years of age are available in the kuru region and, such as there are, are apt to suffer from loss and lapses in their recall and present an appearance and a behavior pattern of premature aging. Thus, were kuru an ancient disease among the Fore, one could conclude *a priori* that attempts to elicit the past history of kuru in the region would result, nevertheless, in obtaining reports of few cases as distant as 35 years ago and very few, if any, reports of the disease for the more remote past. In this culture, poor in legend and myth or verbal epic, the history is confined to mnemic record and this effectively terminates in the 4th decade in the past.

Time scale among natives in the kuru region. As with most other peoples in the New Guinea Highlands, the kuru region natives have no calendar. This is not surprising since they live in a region without winter and summer seasons and where even the rainy and dry seasons are variable in duration and in regularity of occurrence. Thus, the Fore had no concept of solar year until their contact with civilized man during the past few years. It follows that they do not keep track of their chronological ages and that there is no possibility of learning from them even an approximately numerical estimation of their age. Their attempts at judging an infant's or a child's age in years may yield ridiculous estimations. However, adults in the community do have a very accurate mnemic record of genealogical lineage and of the time sequence of births in their community. Thus, although they cannot state the age of anyone, they can consistently order all inhabitants of their villages in a time-of-birth sequence. This accurate and almost universal knowledge in any village permits the construction of a crude time scale. This we do by means of our approximate knowledge of child growth and development patterns among Melanesian children in coastal communities and in a few Highland communities

wherein mission or government birth records have been available.[22] From these studies we are able to estimate the ages of children rather accurately, we believe, and the extrapolation of the data from nearby Highland communities to Fore children is probably justified. Thus, we line up all the children of any village according to our assessment of their ages and refer all events to the time ladder they represent, by such statements as: "Before A (or B, C, D . . . etc.) was born," or, "A few days after E was born," or, "When F was as big as A now is." A similar technique can be used to extend time estimates back for 20, even 30 or 35, years. However, our accuracy of age estimation decreases with increasing age. Rough estimates of our range of possible error and of the consistency of our independent age assessments are shown in Table 2.

TABLE 2

The range of possible error and maximum deviation in the assessment of age for various age groups by different investigators

Assessed age (years)	Range of possible error	Maximum deviation
0–1	3 mo	2 mo
1–4	6 mo	6 mo
5–9	1 yr	9 mo
10–14	1.5 yr	1 yr
15–19	2 yr	2 yr
20–29	5 yr	5 yr
30–39	10 yr	5 yr
40–49	10 yr	5 yr
≥ 50	20 yr	10 yr

Such events as the time of first contact with a government patrol or the time of our first entry into the region to initiate investigations of kuru can be used as a second set of references to aid dating, in addition to the birth sequence scale of time. Important "prehistoric" events have been repeatedly documented by use of the time scale based upon age estimation, and these events can thereafter be used for the construction of "calendars" in the kuru region.

The inadequacy of time estimates based on the absence or existence of systems of sorcery. Frequently the existence of an organized pattern of sorcery and magic to account for a disease has been accepted as tentative evidence for long standing experience with the disease entity. On the other hand, an illness has been adjudged a recent introduction into native communities, probably brought in for the first time with European contact, on the basis of a total absence of the usual systems of sorcery to account for the disease. This is probably a justified method of inference, but it is devoid of any element of certainty. In the region in which kuru occurs the natives assign the etiology of most diseases to specific sorceries. However, some ailments are not so accounted for. Thus, we have found that a large group of diseases designated as "yointana" or, in pidgin English "sik nating" are not attributed to sorcery, and these are certainly not new among the natives. When pressed for further etiological explanation of these diseases, the natives admit they have no explanations; they state that the disease simply arises mysteriously without evidence for malevolent induction or supernatural provocation. Such illnesses are generally the acute infectious diseases of infancy and childhood, acute respiratory illnesses, including pneumonia of childhood, otitis media, meningitis and many other pyrexias of unknown origin. The natives' explanation of these ailments includes acceptance of the ways of nature and recognition that unknown natural causes are involved and a refusal to attribute them to malicious sorcery, to spirits of dead relatives, or to other supernatural magical phenomena. Fatal acute febrile ailments, particularly in infants, are usually so categorized, and nonspecific acute pyrexias at any age may be so accounted for; death of the aged from a wide variety of causes is simply attributed to old age. Thus, long experience with certain disease patterns need not always result in the native mind in the evocation of sorcery as a framework for causeology.

In contrast, an explanation in terms of sorcery may be built up around a disease in a very short period, such as that developed in the Kamano area just north of the kuru region, in response to a newly introduced epidemic dysentery, previous episodes of which were not recalled by the natives.[14] Moreover, we have seen a sorcery explanation assigned to headaches associated with influenza-like epidemic illnesses which followed by a few weeks the venepunctures which we performed throughout the area in the collection of blood specimens from normal individuals. This respiratory disease epidemic was introduced

via the channels of communication opened by European contact. Elaborate procedures for counter-sorcery have been in use against this "new" disease which, to the native mind, was the result of white man's sorcery worked with the aid of the blood collected. Upon explicit demonstration to the villagers that the vast majority of subjects from whom we had drawn blood by exactly similar procedures failed to coincidentally develop a headache or febrile disease during the weeks subsequent to venepuncture and that many of the cases of such complaints were in individuals from whom we had not drawn blood specimens, the natives, in general, promptly abandoned the sorcery explanations which were spreading like gossip or fad through the community. It is, perhaps, naive to suspect that age-old sorcery beliefs can be so glibly abandoned. Such sorcery systems are often logically self-consistent systems of belief. It is seldom possible to influence the axioms on which they are based by logical argument assuming alternative faiths such as that of Western man in the scientific method. However, we have strong evidence that the dramatic and rapid cure of yaws by penicillin therapy has resulted in many localities in the complete abandonment of the profession of its induction by sorcery. We find indications of the disappearance of a belief in this kind of sorcery in the cessation throughout the community of the practice of yaws-inducing sorcery procedures, even by professed sorcerers, with an explanation to us that yaws is NOT sorcery-induced, since it can be cured with penicillin injections. The simultaneous preservation of sorcery practices and explanations for diseases such as kuru, for which we have no cure or palliative therapy, indicates that the native is not trying to conceal from us his basic conviction in sorcery as etiologically responsible for most diseases.

In summary, we have reason to suspect the validity of any conclusions as to chronicity of a disease pattern based upon the existence or absence of sorcery systems to account for it, in view of the observations of: (1) the rapid construction of organized sorcery explanations and practices, under our direct observation, to meet the requirements of what is interpreted as the occurrence of new disease, (2) the absence of sorcery explanation for some illness with which the natives have certainly had long experience, and (3) the occasional abandoning, or near eradication, of well-established sorcery explanations and practices as has occurred in the face of cure by modern medical methods.

THE ORIGIN OF KURU

There are some 31,000 Melanesian natives who live in hamlets affected by kuru. They inhabit about 140 isolated groups of small hamlets. The linguistic and cultural groups of the region are shown in Figure 3. These communities seldom contain more than a few hundred individuals who form a nearly-closed breeding group. Groups of hamlets are allied in warfare, and the isolation of these fighting units within the kuru region from each other is and has been extreme over many generations. Thus, only a small portion of the wives in any hamlet are bought or captured from outside those hamlets allied to it in warfare; and none, or very few, come from groups more than one or two such warring alliances away. This situation provides clear evidence that marriage pools rarely comprise more than several hundred individuals, and never over a few thousand. Each of the such limited marriage pools which exist in the kuru region overlaps only slightly the adjacent breeding groups. There is no evidence that currently, or in the recent past generations, the entire population of the area has been involved in free marriage interchange over the whole region. In fact, throughout the Eastern Highlands of New Guinea, a pattern of rather limited marriage possibilities, restricted essentially to a group of allied hamlets in rather close proximity, has been the rule and it is difficult to imagine a much different pattern in the recent past. Under such circumstances it is unlikely that a kuru mutation arising *de novo* in one member of the community could rapidly become as diffusely spread through all currently isolated sub-units of the population as has been the case, even if it conferred a distinct genetic advantage on its carriers.

The distribution of kuru incidence rates throughout the kuru region has revealed but slight irregularities and no discontinuities. Thus, every hamlet or small group of hamlets in the entire kuru-affected region is known to have had both recent kuru deaths observed by us and a long past history of kuru. In the South Fore hamlets the incidence attains maximum values, while in some hamlets in the northern and western

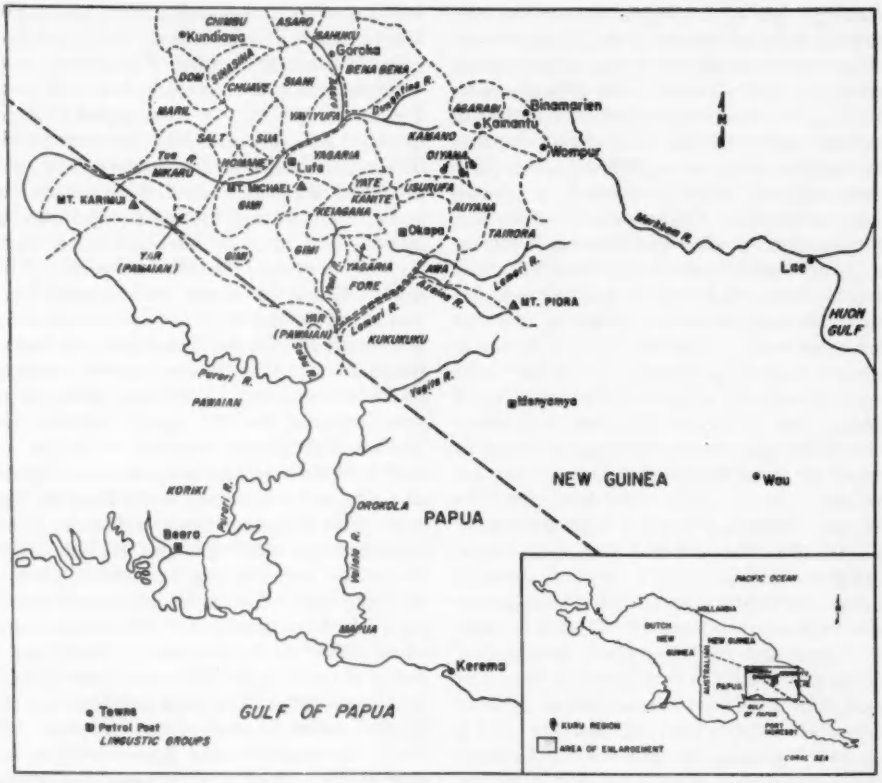

FIG. 3. Cultural and linguistic groups in and surrounding the kuru region in the Eastern Highlands of Australian New Guinea.

peripheral regions it is considerably lower. Since the marriage pattern would make for very slow dissemination of the kuru gene from one small intermarrying group to another, its current wide distribution through all of the far-flung semi-isolated groups of hamlets in the kuru region could only have occurred over the course of many centuries. Thus, if kuru is genetically mediated, it cannot be the result of a very recent mutation. In fact, explanation of the present day distribution of kuru would demand either an unreasonably long estimate of the period the Fore people have been in complete isolation from other peoples, or a different set of hypotheses from those usually applied in the study of the dissemination of a new gene in a human population. At the present time there is no reason to assume that the gene for kuru is at equilibrium level throughout the kuru region, or in any major subdivision of it. On first entry of civilized man into the region, the population was in a state of flux with recent shifts and migrations, and since then conditions have been much further removed from a steady state. Knowledge of the local situation early suggested a means by which a new mutation, associated with a higher genetic fitness in its bearers than in those who did not carry it, such as that for kuru, might rather rapidly attain the high frequency kuru now has in a large population of isolated hamlets. Such a means would be present if the kuru region were originally populated from a single pair (or inbred family) of progenitors. We might call this an "Adam and Eve" or "clonal" hypothesis.

The homeland of both the people of the kuru region and the neighboring Kukukuku natives is the rugged mountainous belt between the more densely populated wide valleys of the

central portion of the highlands and the low, flat malarious sago country of the Papuan coastal plain. Between the Highland peoples and lowland Papuans is a belt of country still without population. It is tempting to hypothesize that into this belt of less hospitable uninhabited country, small outcast groups or single families may have penetrated and been the progenitors of new races. Furthermore, the appearance of kuru in a woman still capable of completing her pregnancy, although ill and already a mother, might easily in itself have been the cause for the expulsion of the patient and her offspring from the community to the uninhabited lands to the south. Such an outcast individual, or a small family group of survivors after an enemy raid, could well give rise to a whole "race" which carries a new mutant gene, present in its progenitors, in rather high frequency in all its current population sub-units. Internal strife, leading to the feud-like internecine wars and fragmentation of the descendents into isolated units, would then no longer provide a barrier to the kuru trait, all sub-units having been endowed therewith before social fragmentation occurred. Thus, an attractive "Adam and Eve" hypothesis is suggested according to which the kuru mutation would be expected to have been more-or-less evenly distributed to all sub-groups of the race arising from these original forefathers. Since, under these circumstances, close inbreeding would be unavoidable, even homozygous individuals would be frequent. Since the kuru trait in heterozygous individuals would usually not prejudice an individual's chances of fulfilling his or her reproductive role and since it has been assumed even to confer a genetic advantage on its carriers, the rapid further propagation of the mutant trait would be assured. What the relative genetic fitness of the carrier of the kuru gene actually is with respect to the normal in the kuru region environment, we do not know; it may well be such as to compensate for loss of the kuru gene in unreproducing homozygous individuals, or, if not so, the kuru gene frequency may now be in the process of decreasing. The reproductive advantage necessary under the above "clonal" hypothesis for attaining the currently observed gene frequencies in a given period of time would be smaller than that necessarily associated with the kuru trait if we considered the more usual situation of population genetics, viz., the dissemination of a new mutation into a large population in only one of whose members it occurred.

In this connection a myth of Kukukuku people is of considerable interest. Kukukuku-type people live to the east of the kuru region in a geographical and ecological belt, between the New Guinea Highlands and the Papuan plains, quite similar to the homeland of the Fore. In fact, during kuru epidemiological investigations, the eastern limit of kuru was established at the boundary between the Fore people and the newly discovered group of Kukukuku-type people on the slopes above the Lamari River, who do not suffer from kuru. There is no written record in the kuru region and we have not succeeded in obtaining any suggestion of the past history of the Fore people and the other kuru-afflicted groups from their legends and myths. There has been no systematic collection of myths and folk stories in the Eastern Highlands other than that done by Beatrice Blackwood during her anthropological studies with the Kukukuku natives along the Watut River in 1936 and 1937.[24] She found many versions of a story of the origin of the Kukukuku people which differed from each other in length and in wealth of detail, but which were much the same in their essentials. This local origin myth is not greatly unlike similar myths reported from Papua. In addition, Miss Blackwood was told another story which describes how the country was repopulated after the original inhabitants had been wiped out in a raid. Since the Kukukuku people are the immediate eastern neighbors of the Fore people, and since they live in an ecological belt quite similar to that of the kuru region and immediately adjacent thereunto, this ancient myth of the origin of population in the region may be of considerable significance to the kuru situation, although it is, of course, only conjectural:

Adventures of a Boy and Girl[24]

"The Nauti came and killed our people in times past. One little girl and one little boy went and hid among the sweet potato plants in the garden. The Nauti killed all the rest of the people and took them away. The two children came out. 'It is no use for us to stay here, presently we shall be killed.' They took sweet potatoes, taro, bananas, and filled up their bags, and went away and slept in another place. (This was repeated

twice.) They went on and went and went and went, they built a shelter, and made a little garden for themselves, they brought taro stalks and young banana shoots. They stayed there, until they had finished nearly all their food, only a little remained. Then the girl said to her brother: 'You stay here, I will go and get food.' She brought a big load of firewood, and said: 'Look after the firewood, make a good fire and keep it alight, don't let it go out.' She went to another place, the little boy slept. She went on a little further, the little boy slept. Then she came to a garden and got some food, when she had finished she slept on the road. The little boy slept on, his fire went out. He was hungry, but did not cook himself food. He nearly died. He was a very little boy. The girl went and went and went, and came to their house, and saw that the boy was nearly dead. She cried over him, and gave him a ripe banana, he ate it and was all right again. The two stayed there.

"One day the girl said: 'I shall go and make armlets down by the river.' The two went. They stayed by the river, the girl made armlets. The boy found a dead tree and threw it into the water. The tide caught it and carried it down. The boy went with it, singing:

'Awe marai, yata maurai gaulemo'

(This was chanted both by teller and interpreter, who could not translate the words.)

"The girl jumped up and called to the boy: 'You are a very little boy, who taught you this song?' He sang and sang as he went down the river. The girl got a stone and cut her head (it was explained that she did so because she was sorry the boy had gone away). The boy came up out of the water and followed the girl. The two came up to the house and stayed there.

"The boy grew up to be a man. There were no other men there, so these two were married, and the girl had children, first a boy and then a girl. They were married to each other and had children. Then the mother had a girl and the daughter had a boy, and those two were married. Now there were plenty of people. They cut bamboos and made houses, and made fire. They were great men in the olden days."

Here we have a mythological account of the origin of a New Guinea people who are close neighbors to the communities of the kuru region and who live in very similar geographical circumstances. The account of incestuous brother-sister union and subsequent close inbreeding describes a situation ideally suited to account for the rather rapid attainment of kuru distribution as it is now observed. Only if the kuru mutation were to have occurred early in the history of such a clonal population could we account for its widespread rather uniform dissemination throughout every isolated, rarely intermarrying subgroup of Fore natives. In the peripheral zone to the north and west we currently observe the kuru trait spreading by diffusion through a slow pattern of marriage exchange—a pattern which would have required many millennia to have accomplished the current dissemination of the trait through the Fore population, had it arisen in one individual of a large initial population into which it subsequently diffused. At the peripheral geographical limits of kuru to the east and south, this pattern of marriage exchange with the neighboring Awa, Kukukuku and Yar peoples is very much more restricted (only a very few, rather recent, such exchanges have occurred) and on these peripheries kuru incidence is high right up to the limits of its occurrence.

DISCUSSION

Our previous studies have led to the hypothesis that kuru is a genetically determined, or at least genetically mediated, illness[1, 4] and this has been supported by further more intensive genealogical analysis of the kuru region population.[9, 12-22] However, whatever the simplest hypothetical gene mechanism may be which will account for the observed pattern of occurrence of kuru, we are still faced with the further question of the mechanism of gene action—it is in this direction that further inquiry must proceed. We are directing our studies toward elucidation of the metabolic defect underlying kuru and the biochemical basis of kuru pathogenesis. This assumes that the kuru producing genetic constitution results in one or more abnormalities in enzyme synthesis and that the resulting inborn error of metabolism leads to the neuropathological manifestations of the disease. We have taken even this approach with caution, for certain infections, and toxic and deficiency etiological possibilities are difficult to establish or to exclude. Thus, one is tempted to continue to search for exogenous factors which may account for kuru and not to dismiss the rather

unlikely possibility that genealogical correlations are spurious. Thus, continued quest for a possible microbial etiologic agent, in particular for one of the more elusive types such as a protozoa, helminth (microfilaria and larval migrans), virus or spirochete is fully justified, although current evidence is against this possibility. Exogenous toxins in food, water, smoke, body painting or other items in environment, or, what is much more difficult to establish, a deficiency state or a relative or competitive deficiency state likewise appear remote etiologic possibilities, but cannot yet be fully discarded. However, the current studies of intermediary metabolism aimed at elucidation of the biochemical disturbance underlying kuru should offer an adequate, although indirect, approach in the event that the seemingly unlikely etiologic possibilities are, in fact, the case, for environmental factors ultimately act through the biochemical pathways of the organism. For such an approach, attention must be focused on ethno-environmental factors and on the physiology of the kuru-predestined patient. We can already easily phrase a number of moot questions:

1. What is the biochemical basis of kuru pathogenesis? The diffuse reaction in the nervous system seen in kuru (gliosis and neuronal degeneration) indicates the possibility that the brain parenchyma has been exposed to a toxic agent which has either gained entry by passage of the blood brain barrier or through metabolic accumulation within the brain tissue itself. In the former case an abnormal metabolite or a normal substance accumulated in excess of normal levels may be circulating in the blood, or a defect in the blood brain barrier may be permitting a normal neurotoxic serum component, usually excluded, to reach the neuronal tissues. Such a situation may have extensive environmental dependence, as does the serum level of phenylalanine in phenylpyruvic oligophrenia or serum level of non-protein bound copper in Wilson's hepatolenticular degeneration.

2. What factors are responsible for the very diverse age of onset of the disease? If the hypothesis that the homozygous condition for the partially penetrant dominant kuru gene results in the disease in childhood is correct, we still must ask what is the triggering mechanism which gives rise in the course of only a few months to the rapidly progressing symptom complex in one child at the age of 5, while in another at the age of 10 or 15? Why does the disease occur in one woman at 20, before she has ever been pregnant, in another in her 30's during a normal pregnancy, and in another after menopause at 50 or even 60 years of age? Furthermore, if the heterozygous condition leads to disease in adult life, particularly in the female, but rarely in the male, we must inquire what endocrine factors or hypothalamic-pituitary influences underlie the failure of the kuru trait to penetrate in the heterozygous adult male. Moreover, the biochemical basis of the occasional remissions and exacerbations of kuru demands elucidation.

3. What is the nature of the reproductive advantage which the kuru trait confers upon its possessors in the Fore environment? Without such an advantage, akin to the malarial resistance associated with the sickle cell trait in Africa, loss of the kuru gene through death of homozygous individuals before reproductive age should lead to its diminished frequency in the population.

The epidemiological studies and the laboratory investigations which follow in the subsequent papers of this series have been directed toward the solving of these problems, although none of these questions can yet be definitively answered. We hope that the data presented may contribute toward this goal.

SUMMARY

Kuru is apparently determined by a dominant gene with incomplete expressivity producing fatal disease in childhood in homozygotes of both sexes and in adult life in heterozygous female carriers, but not usually penetrant in male carriers. This simplest genetic hypothesis which will account for the unprecedented situation which kuru presents in New Guinea has not yet been refuted by studies of genealogies in the kuru region or by other human ecological investigations. No outsiders have yet developed kuru on residence in the region. Carriers of the kuru gene must possess a distinct genetic advantage in the region, for unless such carriers were genetically more fit than non-carriers loss of the gene in non-reproducing homozygotes would have prevented its attaining its present high frequency in the society.

The restricted marriage pattern with island-like isolation of small marriage pools would have led to extremely slow diffusion of a new mutant

kuru gene throughout the population unless the mutation was present originally in the progenitors of an *Urstamm* from which at least the central subgroups of the current kuru afflicted population were derived before the near-total marriage isolation of the various groups of hamlets developed.

Environmental variables and the possibilities of infectious, toxic or deficiency factors operating alone or with genetic dependence have not yet been fully excluded. However, the principal investigative approach is the biochemical investigation of intermediary metabolism of kuru patients and normal natives of the kuru region, some destined to die of kuru. Such studies should advance our understanding of kuru etiology and pathogenesis whether or not it is a solely genetically mediated disease.

The problems and methods of epidemiological work in the region are discussed and the ethnic setting of kuru further elaborated. A summary of the age and sex distribution of 804 studied kuru cases is given.

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STUDIES ON KURU

II. Serum Proteins in Natives from the Kuru Region of New Guinea

C. C. CURTAIN,* D. C. GAJDUSEK† and V. ZIGAS‡

In the quest for evidence of a metabolic defect in kuru and in an attempt to genetically define the kuru-affected population by a study of inherited biochemical factors in sera and erythrocytes, sera from kuru victims and control subjects in the kuru region and from surrounding populations have been studied by electrophoretic techniques on paper, in starch-gels and by the moving boundary method. Serum haptoglobin typing has been done and protein chromatography of the serum proteins has been carried out. Hemoglobin from the clots has been examined electrophoretically for the presence of abnormal hemoglobins. Data on blood group genetic factors in the same subjects as well as additional data on serum haptoglobins, transferrins and ceruloplasmins and on the urinary excretion of β-aminoisobutyric acid will be presented in further papers.

This paper presents data on the serum proteins of 85 kuru victims, of 363 other Melanesian natives and of 50 other members of tropical populations. Of the Melanesians who were not kuru victims, 140 belonged to cultural and linguistic groups affected by kuru and 173 to other tribes of kuru-free Highland natives whose lands lie near to the kuru region; 50 natives were from other parts of Melanesia. The data of 380 analyses of sera from various "control" groups are compared with those from 95 analyses of sera of the 85 kuru victims. Such serum protein, including haptoglobin, investigations are now being extended along genealogical lines in the families of kuru victims. The possible relationship of the findings to the kuru problem is discussed.

The various populations from whom specimens have been studied will be discussed in subsequent papers. The locations of the groups with respect to the boundaries of kuru occurrence are indicated on the map in Figure 1.

* Baker Medical Research Institute, Melbourne, Australia.

† National Institute of Neurological Diseases and Blindness, National Institutes of Health, Bethesda, Maryland.

‡ Department of Public Health, Kainantu, Territory of Papua and New Guinea.

MATERIALS AND METHODS

Sera. Blood specimens were collected between March, 1957, and March, 1958, in New Guinea during field investigations of kuru. Most specimens were obtained in sterile vacuumatic venules and brought rapidly by native carriers to the Okapa patrol post where they were refrigerated. Subsequently, they were carried to the nearest airstrip whence they were flown under refrigeration to Melbourne where serum was separated from the clots. Most serum specimens were separated from the clots within 4 to 5 days after collection. Few specimens were unrefrigerated for more than 2 days and during this time shade and evaporative cooling with moist cloth over the containers was used to protect them from excessive heating. As discussed under Methods, control sera so collected and handled showed no electrophoretic abnormalities.

Some non-Fore control sera were obtained from the Pathological Laboratories, Department of Public Health, Port Moresby, and other control specimens were obtained from the collection of New Guinea sera held at the Australian Red Cross Blood Bank in Sydney. The latter had been collected and transported as described above but had been stored at −15°C for 24 months.

Table 1 summarizes pertinent data on the specimens used in these studies.

Buffers. Sodium veronal buffer pH 8.6, I = 0.1 was used in the majority of the experiments.

Owing to a temporary shortage of veronal, some paper electrophoresis runs were carried out in pH 8.6, I = 0.05 veronal buffer. This resulted in lack of resolution of the α_1 globulin. In the calculation of averages and standard deviations, α_1 globulin values, where determined, were summed with the albumin.

Electrophoresis Techniques

Electrophoresis of hemoglobin. Hemoglobin solutions were prepared by shaking the clot from 5 cc of blood with 2 cc of distilled water and 0.5 ml of toluene for 30 minutes at room temperature. The mixture was centrifuged and the clear hemoglobin solution examined by paper electrophoresis in pH 8.1, I = 0.1 veronal buffer, by

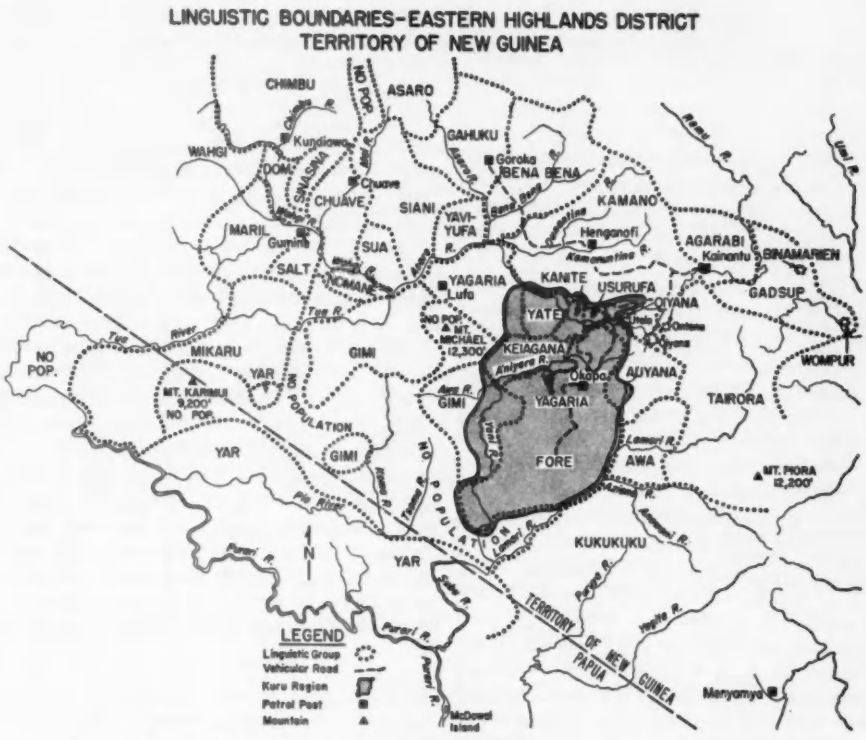

FIG. 1. The kuru region in detail showing boundaries of linguistic and cultural groups

the method of Jencks *et al.*[1] Dr. J. Owen of the Department of Biochemistry, University of Melbourne, confirmed the results by examining 28 of the specimens by the starch-gel electrophoresis technique of Smithies.[2]

Electrophoresis of serum. Moving boundary electrophoresis was carried out in a Perkin-Elmer Model 38A Tiselius apparatus fitted with phaseplate gradient interferometric optics, using monochromatic light at 5461Å.[3] Patterns were evaluated by counting the fringes between the minima of each peak in the gradient curve.

Complex asymmetric peaks were resolved graphically by plotting the reciprocal of fringe width, as determined in a photoelectric fringe counter and measurer (Curtain, to be published), against distance of migration. The raised γ_1 globulin found in many Fore sera was evaluated in this manner.

If, because of slight hemolysis or turbidity, the samples absorbed too much light at 5461Å, an orthodox Philpot-Svennson optical system was used with a 6850Å filter and a tungsten source. These patterns were evaluated by tracing and planimetry. In the case of slightly hemolyzed samples the hemoglobin concentration was determined photometrically at 5400Å and a correction applied to the β globulin values.

Paper electrophoresis was carried out by the method of Jencks *et al.*[1] The strips were evaluated on either an "EEL" photoelectric scanner or a Spinco-Beckman "Analytrol." Hemolyzed samples were corrected for hemoglobin content as described above.

Starch-gel electrophoresis was carried out by the method of Smithies.[2] Haptoglobin typing was facilitated by adding to the sera, before electrophoresis, hemoglobin in a concentration equivalent to 150 mg per 100 ml of serum. After the slabs of gel were cut at the end of the run, as described by Smithies, one-half was stained in the orthodox manner with naphthalene black and the other with the O-dianisidine-hydrogen peroxide stain of Owen *et al.*[4]

Haptoglobin typing of kuru and control sera was considered to be of importance, because it

TABLE 1

Number of specimens studied by quantitative electrophoretic techniques

	Serum protein determinations				Hemoglobin electrophoresis
	Number of			By moving boundary techniques	
	Analyses	Specimens	Subjects		
Groups affected by kuru					
Kuru patients					
in stage I	(17)	(16)	(14)	(2)	(10)
in stage II	(52)	(47)	(46)	(8)	(29)
in stage III	(26)	(25)	(25)	(3)	(12)
Fore	(75)	(69)	(67)	(9)	(38)
Keiagana	(4)	(4)	(4)	(1)	(3)
Gimi	(9)	(8)	(7)	(2)	(5)
Kanite	(1)	(1)	(1)	(0)	(0)
Yate	(3)	(3)	(3)	(0)	(3)
Yagaria	(2)	(2)	(2)	(1)	(1)
Usurufa	(1)	(1)	(1)	(0)	(1)
Total	95	88	85	13	51
"Recovered" kuru	15	15	12	3	7
Congenital tremor	2	2	2	0	1
Aseptic meningoencephalitis	2	2	2	0	2
Normal subjects					
Fore	98	98	98	27	37
Keiagana	24	24	24	12	9
Yagaria	1	1	1	1	0
Mixed parentage	5	5	5	1	0
Groups free of kuru					
In New Guinea Highlands					
Gimi-Mani	34	34	34	10	6
Auyana	8	8	8	2	0
Kamano	5	5	5	1	0
Awa	1	1	1	1	0
Kukukuku-Moraei	6	6	6	6	0
Kukukuku-Menyamya	112	102	102	16	0
Chimbu	17	17	17	5	0
Europeans at Okapa	6	6	6	2	0
In coastal regions of Territory of Papua and New Guinea					
Bougainville	27	27	27	28	0
Pacific Islands Regiment	23	23	23	5	0
In tropical South America					
Peruvian Amazon	44	44	44	0	0

has been shown that the haptoglobin groups correspond to three genotypes produced by a single pair of allelomorphic genes.[58]

All type I haptoglobin sera with added hemoglobin were also examined by electrophoresis in pH 6.8, I = 0.05 phosphate buffer on Whatman 3-mm paper. After drying for 30 minutes at 105°C the strips were stained with the O-dianisidine-hydrogen peroxide stain. This is a sensitive method of detecting the presence of very small amounts of haptoglobin in individuals who would have otherwise been typed "O–O" (J. Owen, personal communication).

Badly hemolyzed samples of serum were

examined in starch-gels made with pH 6.5, 0.075 M cacodylate buffer and connected to electrode vessels containing pH 8.6, 0.025 M borate buffer through filter paper wicks saturated with the same borate buffer. This avoided the excessive spreading of the strong hemoglobin band which is apparently due to the interaction of hemoglobin and borate ions in Smithies' gels (Curtain, to be published). The haptoglobin-hemoglobin complexes migrate into the borate gel where they are sharply resolved, while the hemoglobin migrates slowly backwards from the point of insertion.

Because of some difficulty in resolving 1-2 from 2-2 haptoglobin types in some older sera, all specimens giving such difficulty were retyped using Poulik's tris-borate technique and also by Smithies' vertical technique,[5b, c*] both of which methods give greatly enhanced resolution over the older techniques. This served to clearly determine certain 1-2 specimens which were previously difficult to distinguish from 2-2 patterns.

Protein concentrations. Protein concentrations were determined by measuring the refractive increment of the solutions at 5461Å and 20°C.

Protein Chromatography

Protein chromatography was carried out on seven samples of kuru serum and six samples of normal Fore serum. The adsorbent used was diethylamino-ethyl (DEAE) cellulose and the elution schedule described by Sober *et al.*[6] was followed. Two grams of the adsorbent was packed into 33 × 0.9 cm columns. One milliliter of serum was applied to the column and the optical density at 280 mμ of the effluent from the column was continuously recorded by means of a Beckman Model "D.U." spectrophotometer coupled to a chart recorder.

RESULTS

Hemoglobins

Those specimens examined for abnormal hemoglobins are indicated in Tables 1 and 2. In the hemoglobin specimens from 51 kuru patients no electrophoretic abnormality was demonstrable in pH 8.6, I = 0.1 veronal buffer. Starch-gel electrophoresis of 28 of these specimens also failed to reveal abnormal hemoglobins. Likewise, no abnormal hemoglobin could be found in 6 specimens from non-kuru subjects.

* Kindly carried out by Dr. R. L. Kirk, Department of Zoology, University of Western Australia.

Serum Proteins

Moving boundary and paper electrophoresis. Results of serum protein analyses on sera from kuru patients have been tabulated individually in Table 2. Data for studies by the moving boundary technique are given in bold face type. In Table 3 the average values and standard deviations of such determinations on sera from kuru patients and from all other groups have been summarized.

To determine the effect of collection on serum protein patterns, blood was obtained from six Europeans at Okapa patrol post and was transported to Melbourne under the same conditions as were the kuru and control Melanesian samples. Without exception the Europeans gave serum protein electrophoresis patterns within the limits accepted for normal Europeans (Table 3).

Melanesian norms have not yet been established. Since environmental, cultural and racial variety in New Guinea is extreme, a number of different "control" groups have been studied. Thus, specimens from normal natives of the Fore tribe and other tribes in the kuru region have been examined, in order to establish a base line of normal values with which to compare the results on specimens from kuru victims. Since "latent" kuru cases or individuals with the appropriate genetic constitution for kuru may well be present among the normal individuals in the kuru region, such control examinations must be further compared with the results on natives from surrounding linguistic and cultural groups, or portions thereof, who live near to the kuru region but are just outside the limits of kuru occurrence. Finally, these results must be compared with those from Melanesians elsewhere and from other primitive tropical populations. Such a comparison has been made in Table 3.

The great variation in average values of serum protein components between various groups is immediately evident. This variation clearly reflects the necessity for further work on determining the environmental and the genetic (racial) variables which underlie these differences. Because of it the combined average values for all kuru region natives and such averages for residents of kuru-free regions in the Highlands and on the coast are of little significance. There is, therefore, no more satisfactory "control group" with which to compare the serum protein patterns of kuru victims than the Fore natives themselves, although many of these control subjects

TABLE 2

Serum protein patterns of kuru patients

Patient	Sex	Age	Linguistic group	Protein concen. g%	Electrophoretic analysis*						Haptoglobin type	Hemoglobin
					γ	β	α_2	α_1	Alb.	Starch gel		
Stage I of kuru												
Amakiora	F	6	Fore	8.0	30	19	15	6	30	C	1-1	+
Aoga	F	11	Fore	7.1	21	11	7	3	58	A	1-2	+
				7.2	15	12	8	5	60			
Ara (Warebu)	M	25	Fore	7.9	30	26	7		37			+
Aravaiya	F	20	Fore	7.9	22	23	10	5	40	A	1-2	
Ese	F	25	Fore	7.1	25	38	6	4	27	C	1-1	+
Iai (II)	F	30	Gimi	8.7	**15**	**23**	**6**	**10**	**46**			+
Kairosafina	F	18	Kamite	7.9	10	30	25	5	30	B	1-1	
Mare'einamu	F	35	Gimi	7.3	20	38	6	4	32			
				7.3	21	35	5	4	35	C	1-1	+
Mono	F	40	Gimi	8.1	(24	34	14	2	26)			+
				7.9	(**27**	**35**	**10**	**3**	**25**)			
Namaorufa	F	40	Keiagana	7.3	27	18	12	7	36			+
Natameia	F	9	Fore	8.5	39	12	13	7	29			
Nata	F	8	Fore	8.1	31	12	7	5	45	A	2-2	
Noga	F	25	Fore	8.6	23	18	6	6	47			+
Utavuna	F	22	Gimi	7.9	5	20	25	8	42			+
Tawasa	F	14	Fore							C	1-1	
Wani	F	40	Fore							B	1-2	
Stage II of kuru												
Amosere	F	25	Fore	7.8	18	30	7	11	34	B	1-1	+
Aoga	F	11	Fore	8.2	28	24	12		36	C	1-2	+
Asisi	M	11	Gimi	8.3	10	33	7	7	43	C	1-1	
Aso	F	13	Fore	7.7	15	25	10	10	40	C	1-2	
Asomeia	F	25	Fore	7.8	25	19	8	10	38	E	1-2	+
Atogori	M	23	Fore	8.4	(22	34	9		35)	C	1-1	+
				8.5	(**23**	**34**	**8**	**3**	**32**)	C		
Atona	F	7	Fore	7.6	14	26	8	2	50	C	1-2	
Atuna	F	25	Yate	8.1	27	15	14		44			+
Aveto	F	35	Fore	8.1	26	33	5	5	31	C	1-1	+
Awani	F	40	Fore	7.6	32	25	8		35	C	1-2	+
Ereio	M	14	Fore	8.1	14	32	5	4	45	C	1-1	+
Ewanta	F	23	Fore	7.6	20	18	12	6	44	B	1-2	
Gotu	F	30	Fore	7.4	(20	17	8	5	50)	A	2-2	+
				7.6	(**15**	**15**	**12**	**6**	**52**)			
Haiada'abo	M	11	Gimi	7.7	20	14	6	5	55	A	1-2	+
Hime	F	20	Keiagana	7.6	**15**	**21**	**6**	**8**	**50**			+
Iai (I)	F	25	Fore	8.2	10	20	15	10	45	A	2-2	+
I'amu	F	7	Yagaria	7.3	26	14	14	6	40	D	1-2	+
Iamogi	M	17	Fore	7.7	10	25	25	5	35	B	2-2	+
Inome	F	6	Fore	7.8	8	27	10	10	45	C	1-2	
Inare	F	30	Fore	6.9	25	15	8	8	44	E	1-1	+
Isoisi	F	6	Fore	7.1	**15**	**29**	**10**	**15**	**31**			+
Kaiao	F	45	Usurufa	8.1	8	28	5	5	54	C	1-1	+
Kagobina	M	8	Fore	7.4	(20	18	10	8	44)	B	1-1	
					(**35**	**12**	**15**	**2**	**36**)			
				7.9	(10	27	15	10	38)			
					(**16**	**25**	**7**	**7**	**45**)			

TABLE 2—*Continued*

Patient	Sex	Age	Linguistic group	Protein concen. g %	Electrophoretic analysis*						Haptoglobin type	Hemoglobin
					γ	β	α₂	α₁	Alb.	Starch gel		
Stage II of kuru												
Kasiwari	M	30	Fore	7.7	23	8	21		48			+
Kik	F	25	Fore	7.9	**21**	**17**	**4**	**6**	**52**			+
Kisago	F	30	Fore	8.5	31	18	16		35	B	1-1	
Kogo	F	30	Fore	8.2	36	19	13	6	26	E	2-2	+
Kumpa	M	14	Fore	7.5	28	13	10		49			
Kuroeva	M	18	Keiagana	7.8	17	44	3		36			
Makare	F	14	Fore	7.6	24	26	12		38	B	1-2	+
Manto	F	30	Fore	7.1	29	15	12		44			+
Mande	F	7	Fore	8.1	28	20	6	6	40	B	2-2	
Meio	F	30	Fore	7.3	5	31	13	6	45			
Mulinapa	F	6	Fore	7.7	(15	28	7	6	44)	C	2-2	+
				8.2	(**13**	**29**	**11**	**7**	**40**)			
Natameia	F	9	Fore	8.2	35	15	18	3	29			
Nata	F	8	Fore	7.3	24	31	10		35			
Noga	F	25	Fore	8.2	37	22	8	5	28			+
Nogonta	F	30	Fore	6.9	10	26	15	10	39	C	1-2	
Pesoritme	F	23	Fore	7.1	29	20	10	5	36	B	1-1	
Tageiro	F	25	Fore	7.7	28	16	16		40	E	1-1	
Taranto	F	40	Fore	7.3	18	15	7	7	53	B	2-2	+
Tereiavi	F	30	Fore	8.8	40	12	10	8	30			
Tete	F	20	Fore	7.3	32	10	5	5	48			+
Tovepi	M	19	Fore	8.5	28	25	10	5	32	B	1-1	+
Yayarimeso	F	28	Yate	7.4	27	17	11		45	E	2-2	+
Yosetme	M	5	Fore	8.4	23	30	4		43	C	1-1	+
Sama	F	30	Fore							E	1-2	
Stage III of kuru												
Aranaka	M	7	Fore	7.9	5	35	12	12	36	E	2-2	+
Auwatnefa	F	30	Yate	8.3	38	12	14	6	30	F	2-2	+
Avie	F	35	Fore	7.9	34	10	8		48			+
E'Eya (II)	F	28	Fore	8.1	(38	15	7	6	34)	E	1-1	
				8.2	(**32**	**10**	**7**	**6**	**45**)			
Eruao	F	25	Fore	7.9	27	19	4	3	47	E	2-2	
Faia	F	45	Keiagana	7.3	32	21	3	5	39			+
Haiada'abo	M	11	Gimi	8.0	25	12	5	6	52			
Inam	M	18	Fore	7.5	18	12	10	8	52			+
Inome	F	6	Fore		29	13	20	7	31			
Intako	F	7	Fore	7.3	22	15	20	4	39	E	1-2	+
Isuyema	M	4½	Fore	7.6	34	17	13		36	F		
Kinao	F	5	Fore	8.3	12	30	10	8	40	C	1-2	
Makare	F	14	Fore	7.9	38	25	8	6	23			
Nene	F	50	Fore	8.3	14	12	8	7	59	F	2-2	
Pitotigi	F	30	Fore	7.6	20	27	13	3	37			+
Popo	M	19	Yagaria	7.9	**35**	**18**	**12**	**3**	**32**			
Seri	M	23	Fore	7.8	25	17	12	4	42			+
Tasiko	F	6	Fore	7.7	27	15	12		46			
Tatubi	F	16	Fore	7.6	29	18	7	6	40			+
Tereiavi	F	30	Fore	7.2	12	24	6	6	52			

TABLE 2—*Continued*

Patient	Sex	Age	Linguistic group	Protein concen. g %	Electrophoretic analysis*						Haptoglobin type	Hemoglobin
					γ	β	α₂	α₁	Alb.	Starch gel		
Stage III of kuru												
Tete	F	20	Fore	8.3	36	17	8	3	35			
Tomasepi	F	35	Fore	7.2	23	28	3		46			+
Waruye	M	8	Fore	8.6	16	35	17	3	29			+
Yaboiotu	F	50	Fore	7.6	34	10	12	8	36	F	2-2	+
Yoeia	F	45	Fore	8.3	**40**	**11**	**7**	**4**	**38**	F	1-1	
Tese	F	40	Fore							C	2-2	
Yani	M	5	Fore							C	1-2	
"Recovered" kuru patients												
Abareri	F	20	Gimi	8.0	5	25	30	10	30			+
Ailaia	F	30	Fore	9.5	34	16	13	6	31	B	2-2	+
				7.8	19	20	5	8	48			
Areta	F	20	Fore	7.6	23	24	9		44	A	1-1	
Esita	F	25	Fore	7.2	15	30	15	3	37			
Fopoto	F	22	Keiagana	7.6	8	35	6	9	42	C	2-2	+
I'idumu	F	35	Gimi	8.1	**10**	**15**	**25**	**12**	**38**	A	1-1	
Iokoio	F	20	Keiagana							A	2-2	
Iyarabo	F	32	Gimi	8.1	17	16	6	7	54	A	2-2	+
				7.3	**20**	**21**	**8**	**3**	**48**			
Kofi	F	32	Fore	8.1	35	19	6		40	B	1-2	
Mara	F	25	Fore	7.3	10	22	10	10	48	C	2-2	+
Omaiyo	F	30	Gimi	7.6	35	15	13	4	33	B	1-2	+
				7.3	27	16	10	10	37			
Waronesa	F	35	Gimi	7.7	8	15	30	5	42	C	1-2	+
Yakona	F	40	Fore	7.8	**15**	**35**	**13**	**6**	**31**	C	1-2	

* Figures in parentheses represent two different analyses on same specimens of serum. Figures in bold face type were obtained by moving boundary electrophoresis.

are themselves genetically destined to become kuru victims later.

In the Appendix to this paper Student t-tests for significance of differences between average serum protein values of various Melanesian groups as summarized in Table 3 are presented. The italicized values indicate that the probability of the difference being a chance phenomenon lies between 0.05 and 0.01 ($0.05 > P > 0.01$), while the bold face values indicate that the probability of chance is less than 0.01 ($P < 0.01$).

The Fore normals are unusual in possessing relatively lower γ globulin levels (average, 24%) than most other New Guinea natives studied. Specimens from other groups had average γ globulin values ranging from 25 to 36%. Low levels, but higher than those of the Fore, are also found among the Kukukukus living to the east of the kuru region.

The Fores are also unusual in that, of the samples examined, many possessed a sharply separated, elevated γ_1 globulin (6–11%; normal European, 1–3%. Figures 2 and 3). A critical examination of the moving boundary patterns of sera from other groups often revealed an asymmetric γ peak (Figure 4), but with the exception of 18 out of 28 sera obtained from Bougainville, none had the "kink" in the leading edge of the γ peak characteristic of many Fore patterns. The Bougainville pattern (Figure 5) is distinctive, and there is evidence from starch-gel studies that the additional peak is a β_2 rather than a γ_1 globulin.[7] The serum proteins, with the exception of those of the Gimi, do not appear to vary with age and sex. In the case of the Gimi 16 females had an average γ globulin value of 32% as against 24.5% for 30 males.

A collection of 44 sera obtained from members

TABLE 3

Average serum protein values of kuru patients and other Melanesian and tropical residents in the kuru region and in kuru-free areas

	Number of analyses	Electrophoretic analyses									
		Total protein		γ globulin		β globulin		α_2 globulin		α_1 globulin + albumin	
		Av.	S.D.	Av.	S.D.	Av.	S.D.	Av.	S.D.	Av.	S.D.
Kuru patients											
Stage I	17	7.8	0.5	22.6	8.0	23.8	9.4	10.7	6.0	42.9	4.5
Stage II	52	7.8	0.5	21.7	8.5	22.4	7.5	10.2	4.8	45.7	8.1
Stage III	26	7.9	0.5	26.7	9.4	18.4	9.5	9.9	5.6	45.1	8.7
All stages	95	7.8	0.5	23.7	8.6	21.5	7.8	10.3	5.4	44.5	8.0
Kuru region natives											
Normal subjects											
Fore	98	7.4	0.8	23.9	4.3	11.5	3.4	9.3	2.9	55.3	6.4
Keiagana	24	7.8	0.4	28.6	5.0	13.1	4.8	7.9	3.1	50.4	7.6
Yagaria	1	8.4	—	36.0	—	10.0	—	4.0	—	50.0	—
Mixed parentage	5	7.2	0.0	27.8	2.0	13.6	1.0	10.8	3.0	47.8	4.9
"Recovered" kuru	15	7.8	0.5	18.7	9.3	21.6	6.8	13.3	8.1	46.4	7.6
Congenital tremor	2	7.4	—	16.5	—	21.5	—	8.0	—	54.0	—
Meningoencephalitis	2	7.8	—	31.5	—	18.0	—	8.5	—	42.0	—
All groups	147	7.5	0.4	24.4	6.1	13.2	5.5	9.3	3.7	53.1	7.5
Residents of kuru-free regions											
a. Highland natives											
Gimi-Mani	34	8.1	0.6	30.1	5.6	15.5	3.9	9.9	3.6	44.5	7.3
Auyana	8	7.4	0.1	25.1	2.0	15.9	2.8	12.3	2.6	46.7	2.7
Kamano	5	7.3	0.0	27.1	5.0	12.9	1.7	9.9	2.7	50.1	5.9
Awa	1	7.6	—	30.2	—	5.3	—	10.0	—	54.5	—
Kukukuku (Moraei)	6	7.6	0.2	26.9	3.2	6.2	1.6	5.3	2.7	60.2	13.7
Kukukuku (Menyamya)	112	7.4	0.5	27.1	8.0	9.3	4.0	5.6	3.0	58.0	11.1
Chimbu	17	7.8	0.6	30.9	3.3	13.8	2.0	7.8	1.9	47.5	3.4
All groups	183	7.5	0.5	27.9	7.1	11.1	4.7	7.0	3.7	53.9	11.0
b. Europeans in Highlands											
At Okapa	6	7.2	0.3	13.4	3.2	11.2	0.8	9.0	1.8	66.4	2.0
c. Coastal natives											
Bougainville	27	7.8	0.1	29.5	3.6	16.7*	4.7*	6.9	1.8	47.0	7.0
Pacific Islands Regiment	23	8.0	0.7	35.5	6.0	11.9	2.4	8.0	2.3	44.6	5.5
All groups	50	7.9	0.5	32.2	5.7	14.5	4.5	7.4	2.1	45.9	6.5
d. Tropical South America											
Peruvian Amazon	44	7.1	0.1	18.5	4.2	12.5	1.6	8.6	1.4	61.4	7.1

* β_2 10.3 ±2.3; β_1 6.4 ±4.2.

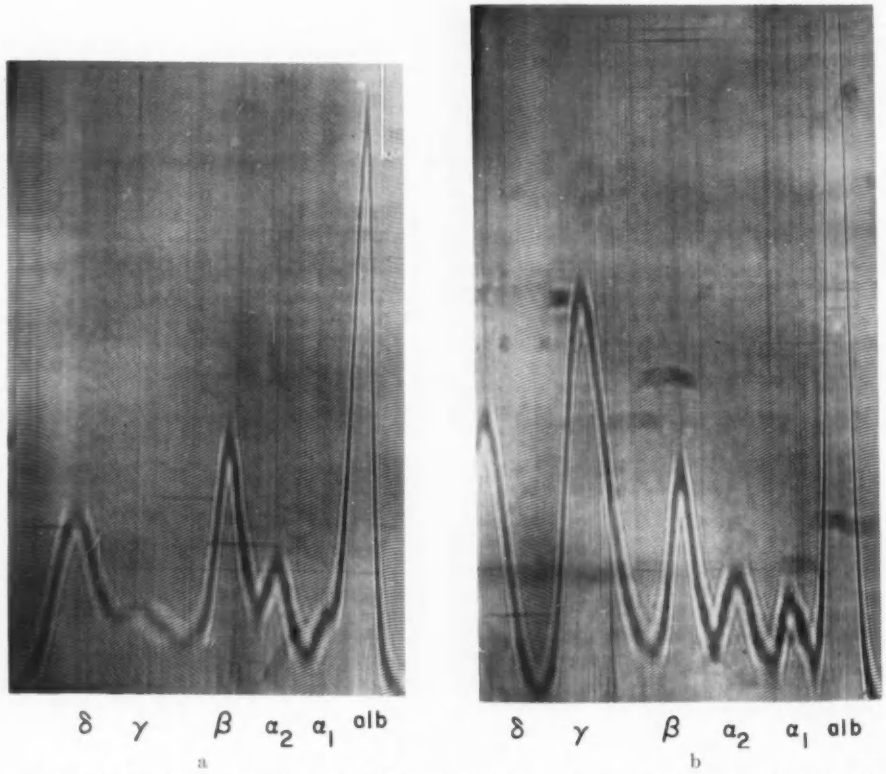

FIG. 2. (a.) Ascending electrophoresis pattern of "high β" kuru serum. Electrophoresis for 3,500 sec at 25 V/cm^{-1} in pH 8.6, I = 0.1 sodium veronal buffer. (b.) Ascending electrophoresis pattern of "high γ" kuru serum. Electrophoresis for 3,950 sec at 25 V/cm^{-1} in pH 8.6, I = 0.1 sodium veronal buffer. Both patterns are phaseplate gradient curves superimposed upon Rayleigh interferometric patterns.

of a group of half-caste Indian-Caucasian people living under primitive conditions in the Iquitos region of Peru (upper Amazon) revealed serum protein levels much closer to the values reported for normal Europeans than to those of any of the Melanesian groups (Table 3). These people live under conditions similar to those of lowland Melanesians, particularly with respect to hygiene, nutrition, climate, clothing and parasitic infestation. The differences in the serum protein patterns between this group and the Melanesians may therefore be a racial difference.

The results of 95 electrophoretic analyses of the serum proteins of 85 kuru patients are presented in Table 2. The disease has been conveniently divided into three clinical stages:[8-13] (I) the non-incapacitating stage of mild ataxia and tremors; (II) the stage of severe ataxia requiring the aid of a stick for locomotion; and (III) the terminal phase of the disease when the patient can no longer walk even with support. It can safely be assumed that the patients in stages I and II of the disease were eating a full normal diet. In stage III all patients lose weight and with increasing dysphagia undergo starvation and later even dehydration. Results are tabulated according to these clinical stages and the patient's age and sex and linguistic and cultural group affiliations are given. When a very small amount of serum was available, the specimen was studied by starch-gel electrophoresis only.

Kuru patients have greater variability in serum composition and average higher β globulin and less albumin than do normal Fore subjects. The β globulin decreases as the disease progresses and the γ globulin increases. High β globulin

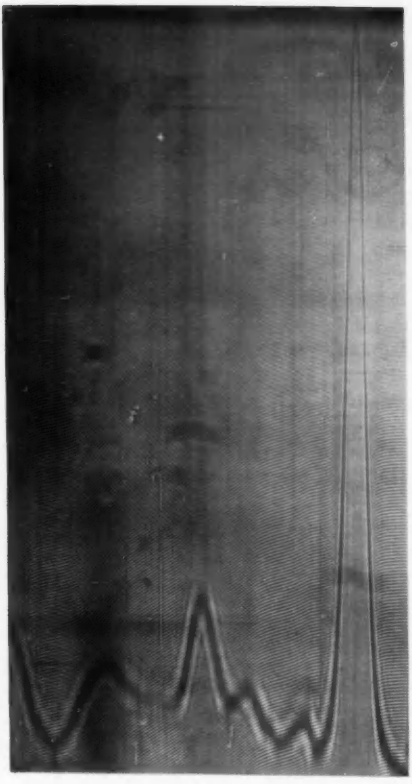

FIG. 3. Ascending electrophoresis pattern of normal Fore serum. Electrophoresis for 3,800 sec at 25 V/cm^{-1} in pH 8.6, I = 0.1 sodium veronal buffer. Phaseplate gradient curve superimposed upon Rayleigh interferometric pattern.

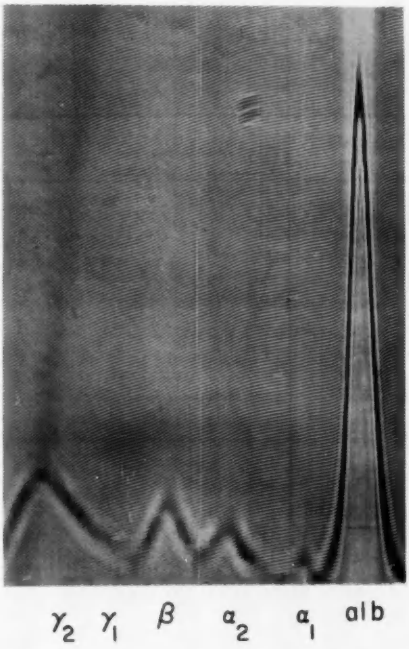

FIG. 4. Ascending electrophoresis pattern of normal Kukukuku serum. Electrophoresis for 4,000 sec at 25 V/cm^{-1} in pH 8.6, I = 0.1 sodium veronal buffer. Phaseplate gradient curve superimposed upon Rayleigh interferometric pattern.

values are particularly frequent in the early stages of the disease. In stages I and II the average γ globulin values are lower, while in stage III the average is slightly higher than in normal Fore individuals. Total proteins are higher in kuru patients than in Fore normals, largely because of the increased β globulins.

The γ globulin in the kuru sera was invariably asymmetrical. All of the elevated proteins gave broad peaks (Figures 2a and 2b) unlike the abnormal proteins of myeloma and lymphosarcoma.

As shown in the Appendix, significant differences between results in stage I and stage II or stage III, or between stage II and stage III, are lacking. However, the raised β globulin and lowered albumin and elevated total protein values in kuru were significantly different from normal Fore values. Of the 95 kuru analyses, 52 showed a high β globulin ($\geq$ 19%) and a slight drop in the absolute concentration of the other serum proteins; 19 analyses showed a high α_2 globulin ($\geq$ 14%) and 23 a high γ globulin ($\geq$ 30%). The drop in γ globulin between stage II and stage III could have been a chance phenomenon with a probability of under 0.05 but not under 0.01. Similarly, the difference in γ globulin level in kuru from that of the normal Fore was not highly significant.

Certain patients have been followed with serial serum examinations. In one, AOGA, a sharp increase was noted in both the γ and β globulins as the disease progressed from stage I to stage II. In another, NATA, the β globulin increased markedly while there was a slight fall in the α

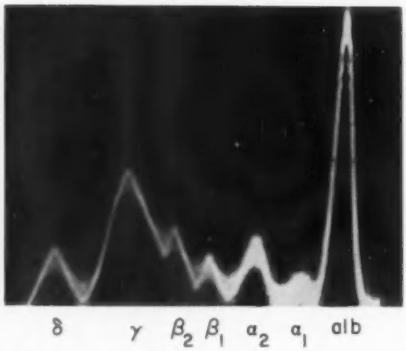

FIG. 5. Ascending electrophoresis pattern of serum from a native of Bougainville, showing the split β globulin. Electrophoresis for 3,950 sec at 25 V/cm^{-1} in pH 8.6, I = 0.1 sodium veronal buffer, with a Philpot-Svennson optical system.

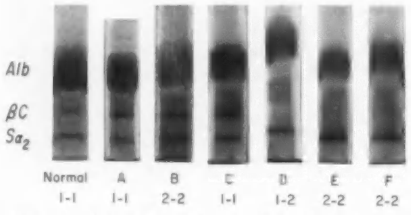

FIG. 6. Starch-gel electrophoresis patterns of normal Fore and kuru sera. The origin is the lower edge of each strip. The γ globulins, which migrate to the rear of the origin in this technique, have been omitted. They were identical in all the sera examined. The letters refer to the classification of each pattern (see text), the figures to the haptoglobin type.

globulin value from stage I to stage II. In two of three patients for whom stage II and stage III sera were available the γ globulin increased markedly and the high β globulin decreased to average normal values (12%). In the third, however, a moderately raised β globulin remained almost constant while there was a sharp rise in the γ globulin. In order to elucidate further this variability the week-to-week changes in the serum proteins of a group of kuru patients are currently being studied.

Starch-gel electrophoresis. Starch-gel electrophoresis gave further evidence of the varied abnormalities in the serum protein patterns of kuru patients. Sera of normal Fore and other Melanesian natives yield starch-gel patterns virtually indistinguishable from those of normal Europeans. In contrast it was found that the sera from 54 kuru patients could be classified into 6 groups (Figure 6). The nomenclature used is that of Poulik and Smithies.[14] In group A (Figure 6A) the pattern was virtually indistinguishable from the normal (Figure 6). In group B (Figure 6B) the βc and $S\alpha_2$ bands were pronounced and there was some blurring of the post-albumin bands. In group C (Figure 6C) the βc band was pronounced. In group D (Figure 6D) the $S\alpha_2$ band was pronounced. In group E (Figure 6E) the $S\alpha_2$ band was pronounced and the prealbumin bands were faint and blurred. In group F (Figure 6F) a belt of many fine bands appeared between the $S\alpha_2$ and albumin.

The patterns show a more consistent correlation with the stage of the disease than do the quantitative serum protein analyses. All stage I patients gave group A, B or C patterns (Figures 6A, B, C); stage II patients gave mostly group A, B or C patterns with a few group D and F (Figures 6D, F). The patterns of stage III patients, with one exception, were all in group E and F. The "recovered" kuru patients had patterns in either group A, B or C, although several had the globulin values found in advance stages of the disease.

Starch-gel electrophoresis is a new technique and relatively little information is available on the type of pattern found in even the more common diseases.

These starch-gel patterns could be correlated with the results obtained by conventional electrophoresis (Table 2). Patients in groups B and C generally had raised β globulin levels. Patients in groups D and E had raised α_2 globulin levels, and patients in groups A and F had either apparently normal patterns or high levels of γ globulin.

Haptoglobin typing. The haptoglobin groups of 80 kuru patients are given in Table 4. These include 52 patients summarized in Table 2 and 28 additional kuru victims. Also, the recent data on haptoglobin types of 47 kuru patients from Bennett and his co-workers in Adelaide[15a] are presented. The number in each group and the gene frequencies are compared in Table 4 with those found for normal Fore and other Melanesian natives, Peruvian natives of upper Amazon, natives of Liberia and the Ivory Coast of Nigeria[15b] and normal Europeans.[16, 17]

The frequency of the Hp^2 gene in the kuru sub-

TABLE 4

Haptoglobin types of kuru victims and of other groups

	Haptoglobin types			Frequency Hp^2 gene	Total number studied
	1-1	1-2	2-2		
Groups affected by kuru					
Kuru patients	31	28	21	0.44	80
Kuru patients (Bennett)‖	21	20	6	0.34	47
Total kuru	52	48	27	0.40	127
"Recovered" kuru	2	4	5	0.64	11
Congenital tremor		2			2
Aseptic meningoencephalitis			2		2
Normal subjects					
Fore	31	17	14	0.36	62
Fore (Bennett)‖	16	15	4	0.33	35
Keiagana	6	2	4	0.42	12
Mixed parentage	3	2	1	0.33	6
Total "normal"	56	36	23	0.36	115
Groups free of kuru					
In New Guinea Highlands					
Gimi-Mani	14	6	6	0.35	26
Awa	6	2	3	0.36	11
Kukukuku-Menyamya	20	30	22	0.51	72
Chimbu	16	9	9	0.40	34
In coastal regions of Territory					
Natives of Bougainville	15	8	5	0.32	28
Pacific Islands Regiment	12	4	7	0.39	23
Non-Melanesian tropical populations					
Peruvian Amazon shore residents (Iquitos region)	13	9	20	0.58	42
Africans (Liberia and Ivory Coast)*	69	60	13	0.30	142
Caucasian populations					
English†	22	121	69	0.59	218
Canadians‡	10	25	14	0.54	49
Australians§	46	116	85	0.58	247

* Data of Sutton *et al.*[15b]

† Data of Allison *et al.*[16]

‡ Data of Smithies.[17]

§ Australian Blood Bank donors; patients attending Alfred Hospital, Melbourne.

‖ Data of Bennett *et al.* [15a]

jects is 0.44 for this series of 80 patients and 0.34 for the Bennett *et al.*[15a] series of 47. The combined data give a frequency of 0.40 which is not far different from the Hp^2 frequency of 0.36 for the 115 "normal" natives of the kuru region.

Allison *et al.*[16] have suggested that the Hp^1-Hp^2 relationship is a true polymorphism maintained, as are those of the blood groups and abnormal hemoglobins, by a balance of selective forces related to susceptibility to disease.[18]

Approximately 1% of the normal sera examined could be classified in the haptoglobin group O–O of Allison *et al.*[16] on the basis of absence of haptoglobin. However where such studies were possible it was found that these individuals had parents and siblings with normal haptoglobin levels.

Further genealogical studies correlated with haptoglobin typing will be needed to clarify this matter.

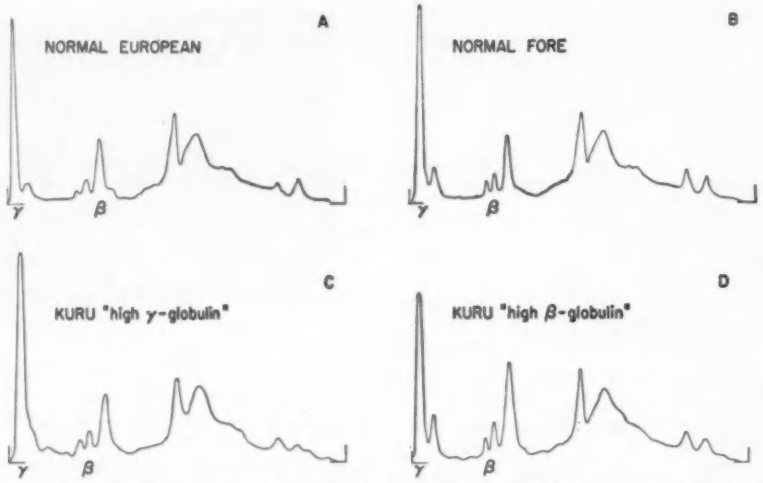

FIG. 7. Chromatography of normal and kuru sera on columns D.E.A.E. cellulose. (A) Normal European serum. (B) Normal Fore serum. (C) "High γ" kuru serum. (D) "High β" kuru serum. Ordinate: $\log_{10}$ optical density at 2800 Å (max 1.0). Abscissa: effluent volume (max 200 ml).

It is of interest that there are relatively minor differences in the frequency of the Hp^2 gene in the Fores and their kuru-free neighbors, although it seems that the frequency of Hp^2 may be lower in other regions of Melanesia.

Protein chromatography. Chromatograms of normal European, normal Fore and "high β" and "high γ" kuru sera are shown in Figure 7. The normal Fore serum (Figure 7B) shows an increase in both the γ and slow β globulin fractions compared with the normal European (Figure 7A). The "high γ" kuru serum (Figure 7C) shows an enlarged γ peak, with some enlargement of the γ_1 peak. The "high β" kuru serum (Figure 7D) shows a considerable elevation of both β globulin peaks.

It is planned to carry out preparative chromatography on larger samples of kuru serum in order to make chemical and immunological studies on the elevated globulin components.

DISCUSSION

The abnormalities in the sera from kuru patients have been detected by comparison of the results with those obtained from sera of normal Fore natives and further with a wide range of normal Melanesian sera. Since in the kuru region appropriate "control" subjects are very likely to be potential kuru victims, the adequacy of such "controls" is subject to suspicion. Therefore, sera from natives from linguistic groups outside the kuru region have been studied as well. For such groups, factors that contribute to serum composition such as genetic stock, nutritional status, parasitic and other infections are inevitably different from those in the kuru region. The ethnic and geographical documentation of these "control" results, however, should render the data of permanent value in future attempts at correlation.

None of the normal Melanesian sera examined gave starch-gel patterns corresponding to the groups B, C, D, E and F found among kuru patients. The particular relationship of these findings to the clinical and genetic features of kuru is not evident. Since kuru appears to be inherited, study of serum protein patterns of kuru-affected families may aid in the determination of whether a genetic factor is involved in the serum composition of kuru victims and may also provide base line values for individuals who may later develop kuru.

The significance of the changes observed in kuru is a matter of conjecture. We have been unable to find any reports of electrophoretic abnormalities in the sera of patients with the classical degenerative neurological diseases.

Zinneman and Rotstein[19] found that β globulin levels were raised and γ globulin levels depressed in dystrophia myotonica, although none of their values approached the extreme found in some

of the kuru patients. There is also some evidence of an increase in serum β globulin after necrosis of muscle (in myocardial infarction).[20] Advanced stages of kuru are accompanied by progressing muscular atrophy which may account for elevated β globulins. It is of interest to note that two cases of aseptic meningoencephalitis showed elevated β globulins. Possibly elevation of the β globulin is a reactive phenomenon exhibited by the Fore and their neighbors in a range of diseases. This possibility is being investigated.

The increased γ globulin found in the terminal stages of the disease is likewise difficult to explain, particularly as autopsy has shown no lymph node or liver involvement. Most authors ascribe high γ globulin levels in the tropics to either chronic infection or to nutritional factors. Holmes *et al.*[21] found correlation between raised γ globulins and the incidence of malaria in Uganda. The relationship of chronic malnutrition (particularly protein deficiency leading to liver damage) to raised γ globulin levels has been stressed by many workers.[22] [23] However, there is no evidence that malaria, chronic infection or malnutrition plays any part in kuru until the late stage when nutritional deficiency first appears. It must be noted also that normal Fore sera are remarkable for their low γ globulin content, compared with the average value for other New Guinea groups. The possibility was considered that the elevated globulins might be auto-antibodies; however, no positive auto-immune complement fixation reactions occurred between sera from kuru patients and normal liver, kidney and brain antigens. These tests were performed by Miss Lois Larkin of the Walter and Eliza Hall Institute, Melbourne, using the method of Gajdusek.[24]

The normal Fore serum globulin profile. There is already much evidence of regional variation in the serum protein profile throughout Melanesia. The present information indicates that the Fore serum globulin profile with its elevated γ_1 globulin is distinct from even that of the Fore's near neighbors, the Gimi and Kukukuku.

Increased values of γ_1 globulin have been found in hyperimmune states in animals, and Blasier and Seitz[25] described γ_1 globulin levels of 6 to 8% in six patients suffering from, respectively, endocarditis lenta, chronic sepsis, cirrhosis of the liver, acute viral hepatitis with chronic cirrhosis, chronic nephrosis and tuberculosis. The Fore are particularly well nourished for a group of Highland Melanesians and kwashiorkor is rare among them. Most of them live in regions free from malaria. Some hobnail cirrhosis of the liver with ascites and death in hepatic coma has been observed among the Fore, but this is a rare condition unrelated to kuru in the area. Thus, nutritional or other factors which might be responsible for the raised γ_1 globulin in the Fores have not been detected.

Haptoglobin typing. Genealogical data on kuru fit well with a genetic hypothesis that kuru is determined by an autosomal dominant gene which, in the homozygous state, produces the disease in both sexes in early life, while in the heterozygous state the trait usually penetrates only in adult females. Thus, such genetically determined species of serum proteins as the haptoglobin, ceruloplasmin and transferrin types are of as much interest as the blood groups in the disease.

The gene frequencies of Hp^1 and Hp^2 in kuru patients were not markedly different from those in normal individuals of the kuru region. The Hp^2 frequencies in the kuru region are rather low when compared with those of most other populations that have been studied. However, Blumberg, Allison and Garry[26] report similarly low values for Micronesians of the Marshall Islands and Barnicot and Kariks'[27] and our data for other Melanesian populations reveal comparably low values.

SUMMARY

Electrophoresis, on paper, in starch-gels and by the moving boundary method, was carried out on the sera of 85 patients suffering from kuru, 363 other Melanesian natives and 50 other members of tropical populations. The results on sera from kuru patients were compared with those obtained for control groups of natives from kuru-affected and kuru-free areas in the Eastern Highlands and from other parts of New Guinea. Extreme variation in serum protein patterns of different ethnocultural groups indicated the necessity for further work on elucidating the environmental and genetic (racial) variables which underlie these differences.

Sera of kuru patients averaged significantly higher β globulin and total protein and lower albumin than did sera of normal Fore natives. The γ globulin levels were generally lower in both kuru victims and normal Fores than in others in Melanesia. In the terminal stages of

the disease there was a tendency towards lowering of the β globulin levels which had become elevated early in the disease. Some terminal patients showed high γ globulin levels.

The electrophoretic pattern of normal Fore natives was distinguished by the appearance of a fairly well separated, elevated γ_1 globulin. This was rarely found in other Melanesian groups, even those closely neighboring the Fore.

Starch-gel electrophoresis in kuru patients fell into 6 patterns which are illustrated and discussed. Normal Melanesians, however, showed starch-gel patterns virtually indistinguishable from those of normal Europeans and not the additional five "abnormal" patterns seen in kuru.

Haptoglobin typing was carried out on 80 starch-gel electrophoresis patterns of serum specimens from kuru patients. The Hp^2 gene frequency of 0.44 was only slightly higher than that among 62 normal Fore natives (0.36). Transferrin and ceruloplasmin and further haptoglobin studies are in progress.

Electrophoresis of hemoglobin was carried out on 51 kuru and 62 normal subjects. In no case could any abnormal components be detected.

Chromatographic analyses of serum protein of normal Fore and of kuru sera are reported.

ACKNOWLEDGMENT

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APPENDIX

Student t-tests for significance of differences between serum protein values of various Melanesian groups

	N	Total protein		γ globulin		β globulin		α_2 globulin		α_1 globulin & albumin	
		$\bar{x}$	t	$\bar{x}$	t	$\bar{x}$	t	$\bar{x}$	t	$\bar{x}$	t
Kuru I	17	7.8		22.6		23.8		10.7		42.9	
vs.			.000		.378		.617		.345		1.340
Kuru II	52	7.8		21.7		22.4		10.2		45.7	
Kuru I	17	7.8		22.6		23.8		10.7		42.9	
vs.			.625		1.449		1.788		.435		.940
Kuru III	26	7.9		26.7		18.4		9.9		45.1	
Kuru II	52	7.8		21.7		22.4		10.2		45.7	
vs.			.833		*2.336*		*2.000*		.242		.297
Kuru III	26	7.9		26.7		18.4		9.9		45.1	
Kuru I	17	7.8		22.6		23.8		10.7		42.9	
vs.			*2.000*		.977		**9.685**		1.489		**7.607**
Fore	98	7.4		23.9		11.5		9.3		55.3	
Kuru II	52	7.8		21.7		22.4		10.2		45.7	
vs.			**3.333**		*2.095*		**12.111**		1.429		**7.869**
Fore	98	7.4		23.9		11.5		9.3		55.3	
Kuru III	26	7.9		26.7		18.4		9.9		45.1	
vs.			**2.941**		*2.188*		**5.847**		.741		**6.623**
Fore	98	7.4		23.9		11.5		9.3		55.3	
Kuru (all stages)	95	7.8		23.7		21.5		10.3		44.5	
vs.			**4.000**		.204		**11.494**		1.613		**10.286**
Fore	98	7.4		23.9		11.5		9.3		55.3	
Fore	98	7.4		23.9		11.5		9.3		55.3	
vs.			*2.353*		**4.608**		1.882		*2.059*		**3.203**
Keiagana	24	7.8		28.6		13.1		7.9		50.4	
Recovered kuru	15	7.8		18.7		21.6		13.3		46.4	
vs.			.000		*2.049*		.047		1.829		.852
Kuru (all stages)	95	7.8		23.7		21.5		10.3		44.5	
Recovered kuru	15	7.8		18.7		21.6		13.3		46.4	
vs.			1.905		**3.537**		**9.018**		**3.571**		**4.837**
Fore	98	7.4		23.9		11.5		9.3		55.3	
Gimi-Mani	34	8.1		30.1		15.5		9.9		44.5	
vs.			**4.667**		**6.596**		**5.634**		.968		**8.120**
Fore	98	7.4		23.9		11.5		9.3		55.3	
Kukukuku-Moraei	6	7.6		26.9		6.2		5.3		60.2	
vs.			.952		.060		1.879		.238		.463
Kukukuku-Menyamya	112	7.4		27.1		9.3		5.6		58.0	
Kukukuku-Total	118	7.4		27.0		9.1		5.6		58.2	
vs.			.000		**3.483**		**4.706**		**9.024**		*2.188*
Fore	98	7.4		23.9		11.5		9.3		55.3	

APPENDIX CONT.

	N	Total protein		γ globulin		β globulin		α_2 globulin		α_1 globulin & albumin	
		$\bar{x}$	t	$\bar{x}$	t	$\bar{x}$	t	$\bar{x}$	t	$\bar{x}$	t
Kukukuku-Total	118	7.4		27.0		9.1		5.6		58.2	
vs.			**7.000**		***2.123***		**8.421**		**6.935**		**6.800**
Gimi-No kuru	34	8.1		30.1		15.5		9.9		44.5	
Kuru region natives	147	7.5		24.4		13.2		9.3		53.1	
vs.			.000		**4.730**		**3.750**		**5.610**		.748
Kuru-free region-Highland natives	183	7.5		27.9		11.1		7.0		53.9	
Bougainville	27	7.8		29.5		16.7		6.9		47.0	
vs.			1.429		**4.255**		**4.324**		1.864		1.304
Pac. Is. Regiment	23	8.0		35.5		11.9		8.0		44.6	
Kuru region natives	147	7.5		24.4		13.2		9.3		53.1	
vs.			**5.714**		**7.879**		1.494		**3.455**		**6.050**
Coastal natives	50	7.9		32.2		14.5		7.4		45.9	
Kuru-free region-Highland natives	183	7.5		27.9		11.1		7.0		53.9	
vs.			**5.000**		**3.945**		**4.533**		.727		**4.878**
Coastal natives	50	7.9		32.2		14.5		7.4		45.9	

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{N_1S_1^2 + N_2S_2^2}{N_1 + N_2 - 2} \cdot \frac{N_1 + N_2}{N_1N_2}}}$$

t = t-test value
$\bar{x}_1$ = Average for Group 1
$\bar{x}_2$ = Average for Group 2
N_1 = Number of analyses in Group 1
N_2 = Number of analyses in Group 2
S_1 = Standard deviation of Group 1 (S_1^2 = Variance of sample 1)
S_2 = Standard deviation of Group 2 (S_2^2 = Variance of sample 2)
Number of degrees of freedom (taken as >30) = $(N_1 + N_2 - 2)$

In italics t = 1.96 to 2.58
In bold face $t \geq 2.58$
Probability $t > 1.96, P < 0.05$
$t > 2.58, P < 0.01$

BOOKS RECEIVED

Control of Communicable Diseases in Man, by the American Public Health Association. 9th edition, 234 pages. New York, American Public Health Association, 1960. $1.00.

Gesundheitstaschenbuch für die Warmen Länder, by Prof. Dr. J. Grober, Dr. H. Horn and Dr. F. Oberdoerster. 1st edition, 332 pages, illustrated. Berlin, Veb Verlag Volk und Gesundheit, 1960. DM 18.80.

Human Toxoplasmosis. Proceedings of the Conference on Clinical Aspects and Diagnostic Problems of Toxoplasmosis in Paediatrics at The VIII International Congress of Paediatrics, Copenhagen, 1956, Revised and Edited 1959, J. Chr. Siim, M.D., Director, The Toxoplasmosis Department, State Serum Institute, Copenhagen, Denmark, Editor. 220 pages, illustrated. Baltimore, The Williams & Wilkins Co., 1960. $12.50.

Handbook of Microbiology, by Morris B. Jacobs, Ph.D., Associate Professor of Occupational Medicine, School of Public Health and Administrative Medicine, Columbia University, and Maurice J. Gerstein, B.S., M.S., Department of Biology, William H. Maxwell Vocational High School, Brooklyn, New York. 322 + x pages. Princeton, New Jersey, D. Van Nostrand Company, Inc., 1960. $8.50.

Medical and Biological Research in Israel, Moshe Prywes, Editor. 562 + xix pages, illustrated. Jerusalem, The Hebrew University of Jerusalem, and Hadassah, The Women's Zionist Organization of America (Grune and Stratton, Inc., New York, distribution agent for the Americas), 1960. $8.00.

Insulin, by various authors, F. G. Young, Editor. *British Medical Bulletin*, volume 16, number 3, pages 175–264. London, Medical Department, The British Council, September 1960. $3.25.

The Urological Aspects of Bilharziasis in Rhodesia, by R. M. Honey, M.B., F.R.C.S. (Edin.), Consulting Urologist, Salisbury Hospitals Group, and M. Gelfand, C.B.E., M.D., F.R.C.P., Consulting Physician, European and African Hospitals, Salisbury; foreword by D. M. Blair, O.B.E., M.D., D.P.H., Secretary for Health, Federation of Rhodesia and Nyasaland. 71 pages, illustrated, paper covers. Edinburgh and London, E. & S. Livingstone Ltd. (The Williams & Wilkins Co., Baltimore, exclusive U. S. agents), 1960. $2.50.

BOOK REVIEWS

Anatomy—A Regional Study of Human Structure, by Ernest Gardner, M.D., Donald J. Gray, Ph.D., and Ronan O'Rahilly, M.Sc., M.D. 1st edition, 999 + xvi pages, illustrated. Philadelphia and London, W. B. Saunders Co., 1960. $15.00.

In this period of radical change in the medical curriculum, the emphasis on human anatomy and the time allowed for its study in the preclinical years have been reduced to such a degree that the large standard systematic treatises on anatomy no longer can be used effectively as required texts. Several shorter texts of anatomy have appeared in recent years with the avowed purpose of filling the needs of the current, briefer courses in the subject. The present volume is the latest of these works to be published, but by no means the briefest.

As stated in the authors' preface, ". . . the MAJOR AIMS of the present work are (1) to provide a textbook that is sufficiently brief for the undergraduate medical and dental student during the present shortened course in human anatomy, (2) to provide information on living anatomy and to stress the importance of the relationship between structure and function, and (3), particularly by the citation of relevant reference to meet the needs of the more advanced student and postgraduate worker." To fulfill these goals there is at hand a text of nearly one thousand pages, profusely illustrated and thoroughly well indexed.

The body of the text is divided into eight parts of which Part One is entitled "General Anatomy." The remaining parts deal with various regions of the body. The ten introductory chapters that comprise Part One serve to describe the major systems of the body, consider development and growth, and outline the principles of radiological anatomy. A discussion of anatomical terminology, a brief history of anatomy and a description of the major sources of reference in the literature highlight the first chapter.

The remainder of the book follows a regional

plan of presentation which the authors hasten to qualify in the preface as "moderate regionalism," stating that ". . . within any given region, however, a rigorous pursuit of the regional method to the exclusion of systemic considerations has not been attempted." Within this framework there are seventy-two additional chapters included in parts devoted to the upper limb, lower limb, thorax, abdomen, pelvis, back, and the head and neck. The intent of the authors is to present a regional approach to conform in general to the manner in which dissection is usually conducted, but at the same time to permit wide latitude in choosing the particular order in which the material may be studied. The text is in no sense intended to be a guide to dissection.

The scope of the work has been kept generally within the bounds of gross anatomy, exclusive of neuroanatomy, histology, comparative anatomy and embryology. Excursions into these related fields have been frequently made, however, if material relevant to a particular discussion is of value.

Critical evaluation of an entirely new textbook may, of course, be tempered in time as experience accrues in the use of the book by students. However, certain initial impressions are strong enough to be set forth here. That this type of book fills a critical need in our present teaching program in medicine and dentistry is unquestionable. That this particular text fills such need with admirable precision is the unqualified opinion of the reviewer. The text is incisive and clear, filled throughout with informative discussions of up-to-date information of the sort that brings the subject of human anatomy to life. These interesting accounts should completely alleviate the perennial complaints of those who find it painful to decipher the minutely detailed descriptions found in older student texts. Yet at the same time there is no apparent loss of essential anatomical information. For the advanced student, the authors have included pertinent references throughout the work.

The illustrations have been designed to accompany the text material and do not in themselves comprise an atlas. Many of the schematic figures are entirely new in concept and require careful study. Others, along more classical lines, ". . . have been drawn from dissections or compared with dissections during their preparation." One notes a certain starkness in the heavy black and white style of the drawings that is not always pleasing. This, together with the rather clumsy label lines in many places, detracts from the total effect of the drawings. Nevertheless, on closer inspection, the important message of each illustration is nearly always abundantly clear. The numerous radiographs that have been reproduced are of excellent quality.

The NA terminology has been used throughout the text with comments on usage being inserted as required by errors or omissions in the official terminology. Eponyms have been completely omitted from the text, in line with the practice of the NA. A useful and interesting glossary of eponyms appears as a supplement at the end of the text.

In summary, here is a text that is refreshing in every detail. It combines discussion of form and function in a manner that cannot fail to elicit the enthusiasm of its readers. The vital importance of anatomy as a foundation in the study of medicine is clearly apparent throughout the work. The book will prove of great value to students beginning the study of anatomy and is highly recommended to those clinicians and advanced students who wish to refresh themselves with concise, up-to-date information.

David L. Bassett
University of Washington School of Medicine
Seattle, Washington

Doctor in Bolivia, by H. Eric Mautner, M.D. 331 + viii pages. Philadelphia, Chilton Company, 1960. $5.95.

Even in this age of wide travel and inveterate tourism, Bolivia remains relatively unknown and unfrequented. Hemmed in geographically, much of the country is still inaccessible and isolated. Underdeveloped politically and culturally, its progress has remained stunted, and but few of its inhabitants have shared in the advantages of the increasing economic status and higher living standards afforded to some of its neighboring countries by the rapid advances of the twentieth century. For generations it has served as a principal source of tin while the remainder of its natural resources have been meager and unexploited. Only a few European settlers, mostly German and British, have been attracted to Bolivia. Their ventures, rendered successful by ambition and industry, stand in towering contrast to the resigned and unenterprising attitudes of the indigenous inhabitants.

The population of Bolivia is concentrated on the altiplano, or high plateau, of the Andes mountain range on the western border. There at 12,000 feet, an elevation exceeding even that of the capital of Tibet, is nestled La Paz, the chief city and functional capital. The mountains slope slowly toward the east where the forested *yungas* and *valles* regions drain into the Amazon and gradu-

ally become the sparsely settled jungles and lowland plains or pampas. Some of the unexplored forests and jungles are inhabited only by hostile, uncivilized Indians such as the Yanaigua which have never been studied anthropologically nor brought under mission influence.

It is in this setting that Eric Mautner begins his account of a sensitive young Jewish doctor who is driven first to France and then Bolivia from his native Vienna by Nazi persecution. In what is obviously an autobiography, Mautner prefers to fictionalize his central figure and call him Martin Fischer. Thus perhaps he could view himself more objectively and possibly take whatever literary license was necessary in the development of some of the interesting incidents and unusual characters which are skilfully and movingly described.

Dr. Fischer is a frustrated and dispirited intellectual who, after a brief sojourn in La Paz, is on his way to serve as health officer in the primitive jungle villages of Santa Morena and Rojas. Mulling over the cruel fate of life, he finds himself a lonely bit of human flotsam, scarred by the past and dubious about the future, and now almost devoid of purpose. While the resumption of former sophisticated comforts and friendships and the need for making a living in the independent practice of medicine appear necessary, he realizes that he is not just seeking asylum, but is consumed with an intense yearning for self-respect and a reason for living.

Martin Fischer never lets himself (or the reader) forget that he is a Jew, a Nazi victim, and, above all, a doctor. Basically and emotionally unprepared for the primitive and isolated life he must lead, he is able, however, because of his excellent medical training and good conscience, to provide the people with the best brand of medicine possible in a mud hut with an untrained assistant and a limited number of drugs. Situations involving feats in obstetrics, major surgery or dentistry (paid for by cows or oxen) and the ingenuity required to serve as medical jack-of-all trades under most primitive circumstances are interestingly recounted. But the story bogs down at times when the author repeatedly cringes as he is overpowered by feelings of inferiority, then compensates with a flaunting of his medical superiority or boastful sexual virility. When he is not crawling at the "wailing wall" in a desperate attempt to "find" himself, he is crawling into the bed of one of the irresistible local belles to forget in repose under the mosquito netting.

The author is a talented writer who has vividly portrayed an array of exciting people whom he encountered during his two years in Bolivia. Among these are an emasculated compatriot from Vienna; a wealthy cattle owner and village patriarch; a wise and pious, though unordained, religious leader; a *curandero* who is a quaint combination of local medicine man, quack and faith healer; several attractive women; and a dog, all of whom have had some overt or subtle influence in the eventual resolution of his problems. Interspersed among these portraits there is much philosophizing on the meaning and order of life and the search for personal identity, but this is at best rather unprofound.

This reviewer, who not too long ago served as a "doctor in Bolivia," but only for several weeks on a special mission, (see this journal, vol. 8, no. 3, p. 372, May 1959) read the book in anticipation of learning much more about the country's medical and socio-economic status than was possible to learn in his own brief but adventurous visit. In this he was disappointed for the information included relates only facts which could have been easily conjectured; namely, that tuberculosis, syphilis and malaria are common problems, while hypertension and duodenal ulcers are scarce in an area in which culture is lacking, newspapers are seen only at intervals of four to six weeks, and travel is contemplated rarely, and then only if urgent. However, not everyone is familiar with the homemade *chica*, a beer-like liquid made from corn, or *aquardiente*, a 100-proof raw distillate of fermented sugar cane, the traditional and usually the only alcoholic drinks available in remote parts of South America.

Dr. Mautner finally gave up his struggle with himself and his environment and left in quest of a new goal. Presumably he is happy now as Director of Physical Medicine and Rehabilitation at a New York hospital. We trust that Martin Fischer has been equally successful in achieving rehabilitation.

Morris Schaeffer
New York City Health Department

Aedes Aegypti: The Yellow Fever Mosquito, by Sir S. Rickard Christophers, C.I.E., O.B.E., F.R.S., I.M.S. (Ret'd.). 1st edition, 739 pages, illustrated. New York, Cambridge University Press, 1960. $14.50.

By reason of their autecology and synecology, *Homo sapiens* and *Aëdes aegypti* developed close interspecies associations which served to the detriment of man and the benefit of the mosquito. During the current century the tables were reversed and the underdog became the mosquito. When its role as a vector of yellow fever had been demonstrated by the scientific method, technology—the handmaiden of science—was applied with ever increasing effectiveness to the destruc-

tion of this insect species. The control of *A. aegypti* is an outstanding early example of "species sanitation" and "species eradication," both practiced at the turn of the century. Entomologists, who previously had concentrated their efforts on other groups of insects, were now motivated by practical considerations to include mosquitoes in their objects of study, for the occasion was instant. During the period from 1762, when *A. aegypti* acquired a species name, to 1900, when its importance in mass human disease was clearly proved, this insect was regarded as nothing more than a pest by the laity and as an object of curiosity by those interested in natural history. The notable exception to these attitudes was first made public in 1881 by Carlos Finlay, whose hypothesis of mosquito transmission of yellow fever was proved correct by the classic and critical experiments of Walter Reed and his associates.

The past sixty years have witnessed a large and fruitful research effort aimed at the yellow fever mosquito. Many of these results are assembled in this book by Sir Rickard, whose purpose is to provide an up-to-date account which (p. xi) ". . . may be of interest and useful as a work of reference to those engaged in work on the mosquito in the laboratory or in the many connections in which *A. aegypti* now has a place."

The scope is clearly indicated on the spine as well as on the title page: "life history, bionomics and structure." For this reason the reader need experience no displeasure on finding only twenty-three pages devoted to medical aspects, including relation to disease, control of the mosquito, and protection from its bites. The author's primary concern is with this insect as an organism and as a species.

Topics which are treated in separate chapters include the following: historical, systematic, bionomical, medical (including control and protection), technique (laboratory), the egg, eclosion, embryology, the larva (6 chapters), the pupa (2 chapters), and the imago (15 chapters). It is unfortunate that, for technical reasons, studies of the embryology of *A. aegypti* had not been performed; the reader should note that the information in this particular chapter is based on Christophers' research on the embryology of *Culex molestus*. In addition to general considerations of the adult, one finds chapters devoted to the external morphologic characters, food and feeding, the blood meal, mating and oviposition, flight, special senses and behavior, viability under various experimental conditions (including insecticides and repellents), internal structure and organ systems, special sense organs, and physiology (including genetics).

Among the numerous noteworthy features of this book are the excellent original illustrations and the meticulous documentation of references. In a work so large and comprehensive as this book, mistakes of omission as well as commission can doubtless be found by a reviewer of sufficient erudition and experience. It is this reviewer's impression that Christophers' work can be characterized as excellent though imperfect, as opposed to those books which are perfect but mediocre. Hence it seems out of place in this review to indulge in Beckmesserian criticism of details.

The reader who perchance is not acquainted with Christophers and his previous work may be interested and perhaps astonished to learn that this book appears in the 87th year of the author's useful, productive and distinguished life of service to mankind. Surely Sir Rickard has succeeded in producing a book which will be consulted frequently by persons concerned with various aspects of biology, as well as by those who require concrete factual information in their work on medical entomology, tropical medicine and hygiene.

HENRY S. FULLER
WALTER REED ARMY INSTITUTE
OF RESEARCH
WASHINGTON, D. C.

Antibiotics Annual 1959–1960 (7th Annual Symposium on Antibiotics), HENRY WELCH, Ph.D., and FELIX MARTI-IBANEZ, M.D., Editors. 1034 + xx pages, illustrated. New York, Antibiotica, Inc., 1960. $15.00.

The proceedings of the Seventh Annual Symposium on Antibiotics are printed in this volume. The meetings were held in Washington, D. C., on November 4, 5 and 6, 1959. At this writing, it appears improbable that these symposia, as such, will be reinstituted. This is not likely to cause many tears to be shed, for the feeling had been growing in many quarters that some sort of a change in structure and conduct was overdue.

The Seventh Symposium was similar in format to preceding ones. The Chairman was Dr. Henry Welch (since resigned from the Food and Drug Administration). A wide range of material is presented in these 1034 pages. The first several papers consist of addresses by Drs. Felix Marti-Ibanez, Austin Smith, Maxwell Finland, William Kessenich and John J. Curry. They are centered around the word "challenge" and range from a discussion of the great historical challenges in medicine to the challenges of new drugs to the pharmaceutical industry, the clinical investigator,

the Food and Drug Administration and the practicing physician. These are followed by more than 100 papers that are concerned with a variety of subjects such as, new antibiotics (colistin, potassium penicillin-152, fervenulin, streptozotocin and rifomycin), some well-known but restudied ones, toxicity data, sensitivity testing, metabolic studies, experiences with antibiotic combinations and the anti-tumor activities of antibiotics. The final two presentations consist of panel discussions, one of which is entitled, "The Present Status and Problems Involved in Human Sensitization to Antibiotics" and the other, "The Chemotherapy of Acute and Chronic Pediatric and Geriatric Infections." The many papers that deal with antibiotics vary, as usual, in quality. Some are brief, superficial notes whose need for publication is questionable while others are of a good enough quality to have been acceptable to the better scientific journals. In fact, this is typical of this annual volume, namely that excellent, solid material often has been lost in a mass of reports that sometimes leaves the impression that publication was for reasons of expediency. It is to be hoped that whatever mechanism, if any, is found to replace these annual symposia, that it will manage to rid itself of ill-defined, poorly controlled "data" and supply, instead, a high-level type of objective report; ultimately, this should prove to be of real value to the laboratory and clinical investigator and, eventually, to the practicing physician.

HARRY A. FELDMAN
UPSTATE MEDICAL CENTER
SYRACUSE, NEW YORK

Parasites and Parasitic Infections in Early Medicine and Science, by R. HOEPPLI, M.D., D.S.C. 526 pages + 23 plates. Singapore, University of Malaya Press, 1959. M$ 25.00. Oxford University Press, 1960. 63 shillings.

While Doctor Hoeppli was Professor of Parasitology at the Peking Union Medical College from 1930 to 1952, he and his Chinese associates gathered material on parasitological subjects from early Chinese medical books and European literature and published several papers in Chinese journals. While he was Visiting Professor of Parasitology at the University of Malaya in Singapore from 1952 to 1958 he extended his historical studies to the islands of the Southwest Pacific and published several papers in the *Proceedings of the Malaya Alumni Association*. The present book is a series of loosely connected essays which includes not only reprintings of these papers, usually with alterations, but also chapters and sections on similar subjects from other eastern and western countries and civilizations from ancient times to the middle of the seventeenth century.

The author presents much of the material in its original form as quotations from original texts. The Chinese quotations are in the Chinese written characters with English translations. Quotations from Greek, Latin and certain modern languages are also given in the original with English translations. This assures the authenticity of the material. The vast amount of research involved is indicated by the bibliography following each chapter, which totals 1121 references.

The book is divided into three parts. The eleven chapters of Part I deal with early conceptions and problems of parasitology, including supposed origin, pathogenicity, symptoms, beneficial effects and treatment of parasites, imaginary and pseudoparasites, and the use of parasites for therapeutic and cosmetic purposes. A chapter on early illustrations of parasites is documented by thirteen plates reproduced from the literature and furnished with explanatory captions.

Part II, in twelve sections, deals with more specialized subjects, including malaria, dysentery, leeches, scabies and lice in Chinese medicine, early views on parasites among the people of southeast Asia and Oceania, parasites in religion, and the supposed relation of the moon to biology, medicine and parasites.

Part III outlines briefly the increase in knowledge of parasites from 1650 to 1850, when modern parasitology began. At the end of the book are three more plates illustrating Chinese concepts of spontaneous generation, acupuncture and charms against disease; six photographs of modern "spirit doctor" and delousing procedures in Borneo; and a final plate of Roman coins related to medicine. There is a subject index and an index of all names appearing in the book.

This is not a book to be read as a narrative or as a unified textbook. It is rather, in the words of the author, ". . . a collection of material for a history of early parasitology." Its great value lies in its presentation of original material, especially that from Chinese literature, and in its documentation resulting from a scholarly study conducted during a turbulent quarter century in China and Malaya. The book is beautifully printed in large type on soft paper. It should be in every library interested in the history of medicine and parasitology.

HENRY EDMUND MELENEY
UNIVERSITY OF FLORIDA COLLEGE OF MEDICINE
GAINESVILLE, FLORIDA

Surgery and Clinical Pathology in the Tropics, CHARLES BOWESMAN, O.B.E., B.A., M.D., F.R.C.S.E., F.A.C.S., D.T.M.&H., Editor. 1st edition, 1068 + viii pages, illustrated. Edinburgh and London, E. & S. Livingstone Ltd. (The Williams & Wilkins Co., Baltimore, exclusive U.S. agents), 1960. $22.50.

One of England's great contributions to modern civilization is the knowledge of tropical diseases accumulated by her students of medicine and surgery. The far-flung empire, the colonial hospitals and clinics, and the one-man health stations in isolated tropical countries have given many of her physicians and surgeons first-hand knowledge of tropical diseases, their diagnosis and treatment, to an extent rare in the United States. It was thus an interesting and rewarding experience to read this book by Charles Bowesman.

Although there are many excellent text books on tropical medicine, this is perhaps the first and only text book on tropical surgery generally available. It was written by a man with a long and extensive experience in the hospitals, clinics, and small health stations in Africa. Surgery in the tropics is like surgery in temperate zones except that diagnosis, and often treatment, is complicated by the prevalence of malaria, amebiasis, hydatid disease, schistosomiasis, tapeworm cysts, sickle cell disease, and helminthic infections. In a temperate climate a low-grade increasing colonic obstruction in an adult is certainly most likely to be a carcinoma, but in the tropics it could well be due to amebiasis of the colon with the formation of an ameboma. Worms in the intestinal tract may cause intestinal obstruction and they may even cause obstruction in the larynx and trachea. The importance of the widespread infestation of the native peoples with worms is vividly presented. Reference to this complicating factor comes up again and again in discussions of diagnosis and treatment. The author apologizes for this repetition, but the reader finds it useful and valuable.

It is certainly unusual in these days when texts in surgery are often of multiple authorship to find a book like this one written by one man and with sections on general surgery, genito-urinary surgery, surgery in obstetrics, and surgery of the eye and of the respiratory tract. Because of the limited supply of specialists the author has had experience in all of these fields and tells of this experience interestingly and well. The book will undoubtedly accompany every surgeon who plans to do surgery in the tropics. It is also recommended for surgeons elsewhere. Within a few hours patients, who have lived long in the tropics and have become sick, may present themselves in clinics and hospitals in any of the major cities. Modern transportation has brought the problems of tropical disease to all physicians.

LESTER R. DRAGSTEDT
UNIVERSITY OF FLORIDA COLLEGE OF MEDICINE
GAINESVILLE, FLORIDA

Laboratory Technique for the Study of Malaria, by PERCY G. SHUTE, M.B.E., F.R.E.S., and MARJORIE E. MARYON, F.R.E.S.; foreword by SIR GORDON COVELL, C.I.E., M.D., D.P.H. 1st edition, 86 + x pages, illustrated. London, J. & A. Churchill Ltd., 1960. 10s. 6d.

After World War I a program was initiated in the United Kingdom to routinely treat neurosyphilitic patients by malariatherapy. The malaria reference laboratory (Horton, Surrey), which was established in 1925 for this program, was placed under the direction of Mr. Percy Shute. During 21 of the past 35 years, he has been assisted by Miss Marjorie Maryon. In the present publication they have recorded some of the knowledge derived from this extensive experience.

They present with clarity and comment a large number of techniques used in the establishment and maintenance of laboratory colonies of mosquitoes and in the study of all stages of malarial parasites in the mosquito and in man. They do not claim to have reviewed all available procedures or that the ones presented are necessarily the best. The techniques described are the ones that have proved successful in their hands. Considering the experience and productivity of these "hands," this should be excellent endorsement for those wishing to use the procedures.

This publication should be very useful to those concerned with the technical aspects of malariatherapy and research. In addition, many of the techniques can be used in the preparation of teaching materials for courses in malariology and parasitology. There is not a stage in the life cycle for which a "time proven" procedure is not described.

Although the authors concentrate on technical procedures, they have fortunately not hesitated to discuss more academic subjects and the epidemiological applications of their laboratory findings. For example, in regard to Ross's black spores they conclude ". . . our observations lead us to believe that Ross's black spores are the result of malaria infection and that if on dissecting a mosquito neither oocysts nor sporozoites are found but the spores of Ross are present either on the stomach, in the thoracic muscles or in the salivary glands, it is evidence of a previous infection." Likewise, in presenting the identification of species of malaria according to the pigment in oo-

cysts, they remind the reader, "Mepacrine may cause the gametocytes of *P. falciparum* to lose their pigment without sterilizing them. Oocysts produced from these pigmentless gametocytes do not, of course, conform to pattern. Therefore in areas where malaria control includes mass drug therapy other than quinine, atypical oocysts may occasionally be seen."

This publication is illustrated by a number of clear photographs and drawings. In addition to a selected bibliography in the back of the book, at the end of each discussion specific references are listed. The book includes both a table of contents and an index.

M. M. Brooke
Communicable Disease Center
Atlanta, Georgia

Clinical Tropical Diseases, by A. R. D. Adams and B. G. Maegraith. 2nd edition, 540 + x pages, illustrated. Oxford, England, Blackwell Scientific Publications, Ltd. (Charles C Thomas, Springfield, Illinois, U. S. agents), 1960. $11.50.

In the introduction to the first edition of *Clinical Tropical Diseases*, the authors express their belief in the need for a handbook giving that information essential for the clinical diagnosis and treatment of those diseases which occur primarily in the tropics. Their aim was to supply essential facts as dogmatically and concisely as they could. The aim in the second edition was to modernize the book without increasing its bulk.

The result is 540 pages of concise information covering forty-four major disease or disease categories which will be of great value to all interested in tropical diseases, but especially those having clinical responsibilities.

The clinical entities are presented alphabetically, each subject usually being described in respect to definition, geographical distribution, etiology, pathology, clinical picture, diagnosis, treatment, and prophylaxis.

The book is both clearly written and pertinent, especially in regard to treatment. It is unquestionably a valuable contribution to clinical tropical medicine.

Vernon Knight
National Institutes of Health
Bethesda, Maryland

The Origin of Life on the Earth (A Symposium), edited for The Academy of Sciences of U.S.S.R. by A. I. Oparin, A. G. Pasynasii, A. E. Braunstein and T. E. Pavlovskaya; English, French and German editions edited for International Union of Biochemistry by F. Clark and R. L. M. Synge. Volume I, 691 + xv pages, illustrated. New York, Pergamon Press, Inc., 1959. $15.00.

Most scientists agree that life had a beginning at some period in the history of this planet. Beyond this area of assent, however, every question is controversial, whether it be the role of nucleic acids in the origin of life, or the causes of the craters of the moon. Still, this is progress from the time, following Pasteur's disproof of the spontaneous generation of microorganisms, when it seemed that one was forced to conclude that life had no origin but, like matter, had always existed. Since then, science has made the important discovery that life is a property of certain molecular combinations, and this knowledge has completely changed our view; for, since these molecular combinations are not eternal, life cannot have existed always. So far has the world come since Pasteur's day that, in August, 1957, a Symposium on the Origin of Life on the Earth was held in Moscow under the auspices of the International Union of Biochemistry. The Symposium (organized by a committee of the U.S.S.R. Academy oi Sciences under the chairmanship of Prof. A. I. Oparin) lasted six days. The present volume of nearly 700 pages records the proceedings of that meeting. Since the present reviewer is one of those who participated in it, he can testify that everything of importance has been faithfully recorded—everything, that is, except the discussions over caviar and beer in the anteroom.

The Symposium is divided into five sections, as follows: 1. "Primary Formation of Primitive Organic Compounds on the Earth," 2. "The Transformation of Primary Organic Compounds on the Earth," 3. "The Origin of Proteins, Nucleoproteins, and Enzymes," 4. "The Origin of Structure and Metabolism," and 5. "The Evolution of Metabolism." The whole is comprised of some sixty-odd papers, together with considerable discussion. Among the authors are V. Fesenkov, H. Urey, J. Bernal, S. Miller, S. Akabori, M. Calvin, L. Pauling, R. Synge, S. Fox, H. Fraenkel-Conrat, G. Schramm, W. Stanley, A. Belozerski, A. Mirsky, J. Brachet, N. Sisakyan, A. Oparin, D. Sapozhnikov, A. Braunstein—and many others. The topics cover a wide range, from the atmospheres of the planets to the evolution of photosynthesis. With a few exceptions, the papers are technical in nature, and the reader should not be misled by the popular-sounding title. The papers, which are rather uneven in quality, encompass a wide variety of facts, opinions, speculations, and surmises.

The reader may or may not learn much about the origin of life from this book, but he can learn

a good deal about Soviet biology. The collection makes available a large number of articles by Soviet authors, mostly in English, covering a broad segment of science. One can get here, more readily than in any other book I know, an impression of the style of contemporary Soviet biology; and a careful reading of the contributions by Russian authors, including the discussions, will be rewarding to the interested reader. One is struck, for example, by the attempts at novel approaches to various problems that are being made by Russian scientists—although one cannot help feeling at the same time that many of these approaches are naively optimistic.

One can also find here an answer to the question of the status (as of 1957) of genetics in the Soviet Union. Modern studies on the structure, replication, mutation, and mode of action of the genetic material come closer than any other contemporary investigations to a direct attack on the problem of the origin of life. As one result of these studies, the laboratory synthesis of a virus is now a foreseeable development. W. M. Stanley, in his paper in this Symposium, goes even farther: "I believe that the elucidation of the structure of nucleic acid in all of its aspects is the most important scientific problem we face today. . . . It is possible that the solution of this scientific problem could lead eventually to the solution of major political and economic problems." One does not have to share this opinion to agree that the Origin of Life without the Gene is Hamlet without the Prince. It is the great weakness of this Symposium that it does not take adequate account of the discoveries of chemical genetics, or of genetics at all. The brief section devoted to nucleic acids contains several excellent papers on tobacco mosaic virus, but the beautiful and important work of the last fifteen years on the nature of DNA and its genetic role in the cell—work which is fundamental to a discussion of the origin of life—is barely mentioned. Mere passing references are made to discoveries which should have been developed in detail as major themes of the Symposium. This is a fatal defect. It throws the Symposium out of balance, deprives it of an essential point-of-view, and gives the proceedings a faded look from which they are not quite rescued by a number of individually excellent papers.

I should like to add one point that has been left out of these proceedings. During the discussion part of the final session, as this reviewer arose to thank his hosts and make a few general remarks, there occurred to him a comment he had once heard from G. W. Beadle that seemed appropriate to the occasion: "It is hard to make a good theory; a theory has to be reasonable, but a fact does not." This was appreciated by the audience, and I am sorry that it was omitted from the minutes.

N. H. Horowitz
California Institute of Technology
Pasadena, California

NOTICE TO CONTRIBUTORS

The Journal welcomes papers which contribute to the purpose of the American Society of Tropical Medicine and Hygiene—the advancement of knowledge of tropical and subtropical medicine and hygiene through investigation, prevention, treatment and control of diseases and deficiencies. It draws upon medicine, biology, public health, nursing, engineering and allied fields of research and application.

The American Journal of Tropical Medicine and Hygiene combines the American Journal of Tropical Medicine and the Journal of the National Malaria Society and therefore has a special concern with the investigation, therapy and control of malaria in all parts of the world, as well as a broad interest in general parasitology and arthropod-borne diseases.

Papers are ordinarily published in the order of reception, which entails a certain delay in their appearance because of the contractual limitation on the number of journal pages available for each volume. Accepted articles which raise no question of scientific priority but for which immediate publication seems important will be inserted in the next available issue of the Journal if the author or sponsoring institution will pay the cost, estimate of which may be obtained from the Editor. The insertion of such articles does not affect publication of other manuscripts awaiting their turn, since the inserted articles constitute additional pages in the volume without additional cost to the subscriber.

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